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The Influence of Spore-forming Microorganisms on the Quality and Functionality of Cultured Dairy Products

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THE INFLUENCE OF SPORE-FORMING MICROORGANISMS ON THE QUALITY
AND FUNCTIONALITY OF CULTURED DAIRY PRODUCTS

BY
DIPAKKUMAR MEHTA

A dissertation submitted in partial fulfillment of the requirements for the

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Specialization in Dairy Science

South Dakota State University

2018

THE INFLUENCE OF SPORE-FORMING MICROORGANISMS ON THE QUALITY
AND FUNCTIONALITY OF CULTURED DAIRY PRODUCTS

This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree in Biological Sciences and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this dissertation does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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I am dedicating this dissertation to my family, especially my mom, Dakshaben S. Mehta who is eagerly waiting for this moment and who had dreamed to become a mom of Dr. Mehta. Mom, I am pretty sure that the day I will defend my dissertation, you are the one who will be the happiest person in the world. I am writing your name here so that it will set up like a stone for forever and you can see your name whenever you see my dissertation. To my dad, Subhashchandra R. Mehta, who always there when I need him. Dad, thanks for your all support. To my loving sisters, Ulka & Payal, who always encouraged me. To my wonderful wife, Deepal as my backbone. To all who came across during my journey at SDSU.

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ABBREVIATIONS

CN	Casein
PBS	Phosphate-buffered saline
EPS	Exopolysaccharides
NCN	Non-casein nitrogen
MFGM	Milk fat globule membrane
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
NDM	Non-fat dry milk
BMA	Basic medium agar
cfu	Colony-forming unit
NPN	Non-protein nitrogen
CE	Capillary gel electrophoresis
LMW	Low molecular weight peptides
OP	Other peptides
α -LAC	α -lactalbumin
β -LG	β -lactoglobulin
β -CN	β -casein
α S ₁ -CN	α S ₁ casein
α S ₂ -CN	α S ₂ casein
κ -CN	κ -casein
γ -CN	γ -casein
Cryo-SEM	Cryo-scanning electron microscopy

WHC	Water-holding capacity
n	Flow behavior index
k	Consistency coefficient
η_a	Apparent viscosity
AOH	Area of hysteresis loop
CSLM	Confocal laser scanning microscopy
G'	Elastic modulus
G''	Viscous modulus
G^*	Complex modulus
Hz	Hertz

ABSTRACT

THE INFLUENCE OF SPORE-FORMING MICROORGANISMS ON THE QUALITY
AND FUNCTIONALITY OF CULTURED DAIRY PRODUCTS

DIPAKKUMAR MEHTA

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Spore formers are observed as the common spoilage-causing microflora in milk and dairy products. Their spore-forming ability increases their survivability during heat treatment which leads to their presence in a final product. Spore-forming bacteria have ability to produce many types of spoilage-causing enzymes which are potential to influence quality and functionality of the final product. Many researchers have studied the ability of spore formers to induce a spoilage in various dairy products but as per our knowledge, their spoilage-causing activities are not described in a detail for cultured dairy products.

In our first objective, we have studied the ability of the common dairy spore formers to degrade milk proteins, fat, phospholipids, common stabilizers, and exopolysaccharides at typical sour cream (24°C) and yogurt (42°C) fermentation temperatures. The ability of spore-forming strains to the degradation of tested components was starch > xanthan gum > protein = gelatin > phospholipid > pectin > fat at 24°C, and starch > protein = gelatin > xanthan gum > phospholipids > pectin > fat at 42°C. Results indicated that exopolysaccharides produced by commercial yogurt and sour cream cultures were susceptible to the degradation by spore formers.

In our second objective, we have studied the influence of proteolytic and lipolytic spore formers on rheology, texture, physicochemical, sensory, and microstructure of sour cream. Spore formers had grown during sour cream fermentation and had influenced its texture by inducing limited level of proteolysis but not shown any lipolytic activity in sour cream. This study indicated that the contamination of proteolytic spore formers during fermentation can bring batch to batch variations in manufacture sour cream.

In our third objective, we have evaluated the influence of proteolytic spore formers on cottage cheese yield, physicochemical, textural, and sensory attributes. Proteolytic spore formers was inoculated in milk and cream dressing. Results indicated lower yield, higher grit, influenced texture for cottage cheese inoculated with proteolytic spore formers, and also observed for bitterness in cottage cheese at the end of shelf life. Cottage cheese mixed with contaminated cream dressing had shown bitterness in cottage cheese without affecting its texture.

CHAPTER 1

Review of Literature

1. Aerobic spore formers in dairy environments

Milk is in a sterile condition when it is present in a healthy udder (Tolle, 1980). Unfortunately, it could be consequently contaminated via different sources from farm to table including external surfaces of animals, feed, during milking, equipment used for milk handling and storage, transportation, dairy processing facilities such as processing lines and equipment, biofilms formed on heat exchangers, use of secondary products as an ingredient, packaging, distribution, refrigeration condition at customers' place. Milk on its natural state is an excellent nutrient growth medium for microorganisms which makes it substantially perishable food item and limiting its shelf life. The excellent nutritional profile, high water activity and about neutral pH of milk provides an ideal environment for the proliferation of microorganisms (Quigley et al., 2013).

Many types of spoilage-causing microorganisms are associated with milk and dairy products, but the spore-forming bacteria are a major concern. Their spore-forming ability allow them to withstand the harsh dairy processing conditions which lead to their proliferation and survival in dairy environments (Logan et al., 2002; Postollec et al., 2012). Spore-forming bacteria are ubiquitously found in the soil as the primary source of contamination (Heyndrickx, 2011), as well as feces, bedding, feed or milking equipment (Gleeson et al., 2013). Additionally, they can enter through raw milk to dairy processing facilities via contaminated teats, milking cups, bulk tanks or via transportation (Pantoja et al., 2011). Spore-forming bacteria are gram-positive can be categorized into 5 different

classes such as *Bacilli*, *Clostridia*, *Erysipelotrichia*, *Negativicutes*, and *Thermolithobacteria* (Galperin, 2013; Zhang and Lu, 2015). Among them, the aerobic spore-forming bacteria such as *Bacillus* and related bacterial species are the most dominant classes which are widely proliferated in the dairy industry. This class of genera is highly associated with spoilage-causing activities and pathogenicity in milk and dairy products (Scott et al., 2007; Ivy et al., 2012). Other than *Bacillus*, the spore-forming related genera including *Geobacillus*, *Paenibacillus*, *Brevibacillus*, and *Anoxybacillus* have also created the research interests in the dairy sector. Some of the species of these spore formers can survive at the higher temperature as well as the refrigeration temperature.

1.1. The genus *Bacillus*

Members of the genus *Bacillus* are gram-positive (sometimes gram-negative or variable reaction), rod-shaped, endospore-forming aerobic or facultatively anaerobic bacteria constitute the family Bacillaceae. The vegetative cells range from 0.5×1.2 to $2.5 \times 10 \mu\text{m}$ in diameter (Turnbull and Kramer, 1991). The optimum growth temperature for the genus ranging from 25°C to 37°C even though some of the members belong to thermophilic and psychrophilic groups which can grow at temperatures as high as 75°C or as low as 3°C respectively. Some of the species of the genus *Bacillus* can survive the extreme low or high pH conditions, ranging from 2 to 10 (Drobniewski, 1993). They are most common genera among the microbial species that can survive the usual heat treatment of milk, grow, and harbor within the biofilms and contaminate the product as it flows through the processing lines and dairy equipment. However, their initial contamination is low ($<10 \text{ cfu/mL}$) but they can survive and grow (germination under

favorable environments) during several steps of dairy products manufacturing including heating or cooling process (Heyndrickx and Scheldeman, 2002). Many species of the genus can be capable of producing the endospores (metabolically dormant phase) that can resist to the unfavorable and stressful conditions such as heat, cold, radiation, desiccation, disinfectants (Turnbull and Kramer, 1991). Many species of the genera are thermoduric in nature which is sub-categorized as thermoduric mesophiles, thermophiles, and psychrotrophs based on the different growth temperature. Thermoduric mesophiles can grow at varying temperature, ranging from 25°C to 37°C, however some of them can grow and survive under extreme condition (Jay, 2012), whereas thermoduric thermophiles can grow optimally at around 55°C as well as survive the pasteurization temperature but their growth temperature ranging from 40°C to 65°C (Scott et al., 2007).

The common *Bacillus* species that found in dairy environments are *B. licheniformis*, *B. subtilis*, *B. cereus*, *B. mycoides*, and *B. megaterium* (Ledenbach and Marshall, 2009), *B. pumilus*, *B. circulans*, *B. coagulans*, *B. megatarium* (Rückert et al., 2004; Buehner et al., 2015; Sadiq et al., 2016), *B. amyloliquefaciens* (VanderKelen et al., 2016), and *B. mojavenensis*, *B. sonorensis*, *B. tequilensis*, *Bacillus atrophaeus*, *B. acarquiensis* and *B. velezensis* (Jeyaram et al., 2011). Some of the *Bacillus* species are closely related to *B. subtilis* with high genotypic and/or phenotypic similarities and therefore, they are not easily distinguishable and considered them as a member of *B. subtilis* group such as *B. licheniformis*, *B. mojavenensis*, *B. pumilus*, *B. amyloliquefaciens*, *B. sonorensis*, *B. tequilensis*, *B. atrophaeus*, *B. acarquiensis* and *B. velezensis*. These species are generally mesophilic in nature. However, *B. licheniformis*, *B. subtilis* and *B.*

pumilus, the most predominant mesophilic species in milk, which are also facultative thermophiles, depending upon the strains (Burgess et al., 2010).

B. licheniformis is ubiquitously present in dairy environments including across the dairy farm as well predominant mesophilic (sometimes facultative thermophiles) species in raw milk (Phillips and Griffiths, 1986). It is facultatively anaerobic, grows at temperatures up to 55°C (Burgess et al., 2010). Thermotolerant strains of *B. licheniformis* are predominating in milk powders (Yuan et al., 2012; Sadiq et al., 2016). The strains of *B. licheniformis* are widely known for their spoilage-causing ability in milk and dairy products by influencing organoleptic and functional properties (Crielly et al., 1994; Dhakal et al., 2014). Many studies have reported that the strains of *B. licheniformis* identified from dairy foods showed extensive genetic heterogeneity which was probably contaminated from the soil as well as sources intrinsic to dairy processing facilities (Rückert et al., 2004; Scheldeman et al., 2005; Dhakal et al., 2013). *B. licheniformis* has the ability to form biofilms in dairy processing facilities, and capable of producing spoilage enzymes, mainly proteases, and lipases (De Jonghe et al., 2010; Reginensi et al., 2011). Some of the researchers have reported that *B. licheniformis* is one of the predominant *Bacillus* species in the dairy industry. For example, a study on the seasonal effects of microbial flora of farms in the Midwest region of USA from bulk tank milk, environment, and corn silage revealed that *B. licheniformis* is the predominant species in summer (49.4%) as well as in winter (62%) among the isolated bacterial species (Buehner et al., 2014).

From the *Bacillus* group, *B. subtilis* is also widely proliferated in milk and dairy products, is strictly aerobic which usually grows at a temperature ranging from 5°C to 20°C, sometimes up to 45°C to 55°C. It is highly associated with spoilage of raw, pasteurized, UHT, and canned milk products (Heyndrickx and Scheldeman, 2002). *B. pumilus* is also strictly aerobic which can grow at a temperature ranging from 5°C to 15°C, up to 45-55°C (Burgess et al., 2010).

1.2. Other spore-forming genera

Spore-forming genera other than *Bacillus* include *Paenibacillus*, *Geobacillus*, *Solibacillus*, *Ureibacillus*, *Virgibacillus*, *Lysinibacillus*, *Psychrobacillus*, *Anoxybacillus* and *Brevibacillus*. Among these spore-forming group, *Paenibacillus* and *Geobacillus* are also as important as the genus *Bacillus*.

Members of the genus *Paenibacillus* belong to the family Paenibacillaceae. The genus *Paenibacillus* was coined by Ash et al. (1993) and proposed that members of ‘group 3’ within the genus *Bacillus* should be changed to the genus *Paenibacillus*. Like *Bacillus* species, *Paenibacillus* species are also considered to be spoilage-causing flora in milk and dairy products. *Paenibacillus* species are predominantly psychrotolerant which can grow at refrigeration temperature (Ivy et al., 2012). Heyndrickx and Scheldeman (2002) mentioned that although a low number of *Paenibacillus* spores can be found in raw and pasteurized milk, they could be potential to cause spoilage in refrigerated products during storage. The most common sources of raw milk contamination by *Paenibacillus* species are silage and feed concentrate for cattle from the dairy farm environment (Vaerewijck et al., 2001; Te Giffel et al., 2002). According to Ivy et al.

(2012), some members of *Paenibacillus* can survive short-time heat treatments above 100°C. Furthermore, even though they present in low numbers in raw and pasteurized milk, they can grow and reproduce in high numbers during refrigerated storage and thus limit the shelf life of milk. In a particular study, *P. polymyxa* was identified as the main spoilage-causing microorganism in Swedish and Norwegian pasteurized milk refrigerated at 5°C or 7°C respectively (Ternström et al., 1993).

In the dairy industry, the thermophilic *Bacilli* are usually enumerated at 55°C. Furthermore, thermophilic *Bacilli* can be divided into two groups: the obligate thermophiles and facultative thermophiles. The obligate thermophiles can grow only at elevated temperature, ranging from 40°C to 65°C which includes mainly *Geobacillus* species. The genus *Geobacillus* was introduced by Nazina et al., (2001) by reclassifying of the thermophilic species in rRNA ‘group 5’ within the genus *Bacillus*. *Geobacillus* species are widely accepted as the contaminants where elevated temperatures prevail during the manufacturing process such as milk powders manufacturing. Some of the strains of *Geobacillus* species can survive the industrial pasteurization temperature of milk and subsequently, their spores adhere to the surface and germinate to form biofilms, resulting in spoilage of dairy products (Murphy et al., 1999). In a particular study, a survey of thermophilic *Bacilli* in milk powders from different countries revealed that *Geobacillus stearothermophilus* is one of the proven problematic *Geobacillus* species in milk powders across the world (Rückert et al., 2004).

1.3. Sources of contamination

1.3.1. At the dairy farm

The incidence of spore formers and their spores in dairy environments is from many sources, beginning from the dairy farm environment itself. The unhygienic practices in the farm environment are one of the potential reason for the contamination of raw milk. In the farm, the primary sources of raw milk contamination are exterior of the udder, equipment surfaces in contact with milk, inadequate cleaning, and sanitizing protocols and other miscellaneous factors (Schmidt, 2008). Other researchers have also reported that the origin of spore formers could be from the soil, feces, bedding, feed, milking equipment or contaminated raw milk via contaminated teats, milking cups and bulk tanks (Heyndrickx, 2011; Gleeson et al., 2013). Moreover, spore counts have sometimes reached up to 3 logs in bulk milk tanks, teat cups and milking clusters (Scheldeman et al., 2005; Vissers et al., 2007). Furthermore, contamination of spore formers can occur during milk transportation as well from farm to dairy processing facilities (Pantoja et al., 2011). Many studies have reported that soil is the major harboring source of spore formers, leading as the primary source of contamination of raw milk (Novak et al., 2005; Hong et al., 2009). According to Slaghuis et al. (1997), aerobic spore count spiked up to log 5 to log 7 cfu/g in the soil which could be subsequently add-up a higher load of spores in raw milk.

1.3.2. At the dairy processing facilities

Raw milk is considered to be a primary source of contamination of spore formers at dairy process plant facilities. As we discussed above, raw milk can contaminate from dairy farm facilities, during transportation or storage at dairy processing facilities. After contamination, sometimes their numbers spiked beyond log 5 cfu/mL in raw milk before pasteurization (Mikolajcik and Simon, 1978). Phillips and Griffiths (1990) have reported that spore formers can be capable of contaminating milk after processing (post-pasteurization contamination). Furthermore, their spores can be activated immediately after heat treatment and subsequently, their germination and growth in milk and dairy products, leading to cause spoilage in the final product. Additionally, contaminated equipment and poor handling within the dairy process plant also serve as sources of contamination of spore formers at dairy processing facilities (Burgess et al., 2010; Faille et al., 2014).

Use of secondary dairy products such as milk powder for the formulation of other dairy products can be served as a source of contamination of spore formers at dairy processing facilities. In good quality of raw milk, spore counts are relatively lower about 50 cfu/mL. During manufacturing of milk powder, milk is concentrated approximately 10 times which results in the concentration of spores with spores count about 10 times higher (approximately 500 cfu/g) in milk powder after drying without considering their growth during processing (Rückert et al., 2004). According to Scott et al. (2007), evaporators and regeneration sections of heat exchangers where the operating temperature remains in the range of 45°C to 75°C, which supports the growth of

thermophiles and their counts sometimes spiked up to log 6 cfu/g in manufactured milk powder. Use of contaminated milk powders as an ingredient during the manufacturing of other dairy products can influence the final product quality.

One another source that could be the potential source of contamination of spores and spore formers in dairy environments is biofilms. The formation of homogeneous or heterogeneous multicellular bacterial communities on the surface of processing lines, equipment, and heat exchangers in the form of biofilms is the major concern for the dairy industry and can be served as the potential sources of bacterial contamination in milk and dairy products. A typical biofilm is composed of extracellular polymeric substances such as organic molecules and polymers which are attached to a surface, embedding vegetative live or dead cells and spores in the form of micro colonies (Burgess et al., 2009). The complex process of biofilms formation and dissemination is responsible for the proliferation of spoilage or pathogenic microorganisms in the form of detached clumps and clusters (Hall-Stoodley and Stoodley, 2005). Biofilms are considered to be a harboring site for spoilage and pathogenic microorganisms which provide defense against the harsh environments (Jefferson, 2004). According to Burgess et al. (2014), biofilms are the reservoir of spores and spore formers, which act as the main contamination source of final dairy products. Some of the *Bacillus* species can produce highly hydrophobic spores that can adhere to the surfaces such as stainless steel and polymers which are commonly used in food processing, and germinate to form biofilms (Wiencek et al., 1991; Husmark and Ronner, 1992). For example, some of the species of *Geobacillus* can survive the pasteurization step during processing and subsequently, their spores adhere to surfaces and germinate to form biofilms, leading to cause spoilage in dairy products

(Murphy et al., 1999; Seale et al., 2012). Many studies have reported that dead ends, corners, crevices, cracks, gaskets, valves, and joints are the suitable sites for biofilms formation (Storgards et al., 1999a,b; Bremer et al., 2006). Dried dairy ingredients such as milk powder are likely to be less prone to the proliferation of microbial growth due to the lower water activity, but they can be served as the contamination source of thermophilic or thermophilic spore formers for other dairy products when they are used as an ingredient. The vegetative cells and spores of these microorganisms are capable of surviving pasteurization during processing and form biofilms on the stainless-steel surfaces of heat exchangers and evaporators (Flint, 1997). The most common aerobic thermophilic and thermophilic spore-forming bacteria from the dairy environments which were frequently identified in dairy biofilms including *B. licheniformis*, *B. cereus*, *B. pumilus*, *B. coagulans* and *G. stearothermophilus* (Flint et al., 1997a; Murphy et al., 1999; Parkar et al., 2001; Scott et al., 2007; Burgess et al., 2010; Shaheen et al., 2010; Yuan et al., 2012).

1.4. Spore formation, germination, and growth

The most important states of spore-forming bacteria are, i) vegetative form: In this state, microorganisms are metabolically active and uses nutrients from the medium for their survival and growth. The other state called, ii) spore form: In which microorganisms undergo the sporulation process in a starving condition which is a metabolically dormant phase of bacteria. Spore-forming ability is the key factor of the genus *Bacillus* and related genera for their survivability to harsh environments in the

dairy processing plant, whereas their germination and growth are associated with spoilage and pathogenicity in dairy products.

1.4.1. Formation of bacterial spores

Sporulation is considered to be a complex phenomenon, generates two distinct cell types: a forespore and a mother cell. Sporulation occurs in a series of several gene expression and produces the matured spore. In general, there are more than 600 genes that are expressed during sporulation process (Graumann, 2012). The fundamentals of sporulation were comprehensively studied on *B. subtilis* as a model microorganism. In addition, the process of sporulation in most of the spore-forming bacterial species follows the same sequence of morphological modifications (Henriques and Moran, 2007). As shown in Figure 1, the basic and most familiar mode of sporulation involves an asymmetrical cell division that leads to the formation of a mother cell and a smaller forespore. Unique transcriptional programs within these cells result in distinct fates for the forespore and the mother cell. For example, the initiation of sporulation in *B. subtilis* is triggered by a lack of nutrients and high cell density. The decision to sporulate is tightly regulated because this energy-intensive process serves as a last resort for these starving cells. In the early stages of sporulation, gene regulation mainly depends on the stationary-phase sigma factor σ^H and the master transcriptional regulator Spo0A. Activation of Spo0A in *B. subtilis* is governed by a phosphorelay system involving several kinases, each of which transmits information about cell condition and environmental stimuli to determine the phosphorylation state of the intracellular pool of

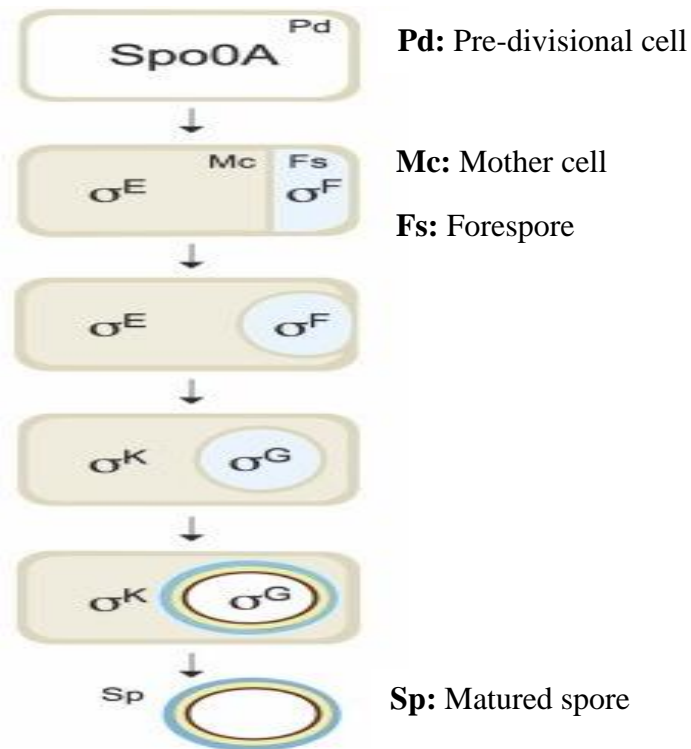


Figure 1. Main morphological stages of sporulation and gene regulation. (Adapted from Chary et al., 2010)

Spo0A. Prior to asymmetric cell division, the chromosome replicates, and each replication origin rapidly migrates to a different pole of the cell. Subsequently, the proximal origin regions become tethered to opposite poles, and the chromosomal DNA stretches from one pole to the other to form an axial filament. During division, only 30% of the origin-proximal portion of one chromosome is trapped within the forespore, and the rest is trans located into the forespore by SpoIIIE, a DNA transporter protein. The other chromosome copy remains in the mother cell (Kay and Warren, 1968). Differential activation of sporulation-specific sigma factors in the mother cell, and the forespore manages the fate of each cell. First, σ^F is activated exclusively in the forespore. Thereafter, a signal is sent to the mother cell to process and hence, activate σ^E . Both early sigma factors promote the expression of genes necessary for forespore engulfment,

as well as genes needed for the production and activation of the late sporulation sigma factors. Remodeling of septal peptidoglycan allows migration of the mother cell membrane around the forespore. Eventually, the leading edge of the migrating mother cell membrane meets, and fission establishes the double-membrane-bound forespore within the mother cell. Completion of forespore engulfment, combined with further intercellular signaling, allows activation of σ_G in the forespore and the subsequent activation of σ_K in the mother cell. These sigma factors regulate the genes necessary for spore maturation and germination. Ultimately, the mother cell undergoes programmed cell death and lysis, which releases the mature endospore (Errington, 2003). Following its formation, an endospore can remain dormant and can persist in unfavorable environmental conditions without moisture or nutrients due to the protective structure and properties of the endospore.

1.4.2. Bacterial spore germination and growth

Under the stressed condition like nutrient deprivation, high temperature, low pH, irradiation, chemical exposure, enzymatic degradation, desiccation, dry and wet heat, UV radiation, mechanical agitation, γ -radiation, chemical exposure, and hydrostatic and osmotic pressure (Nicholson et al., 2000; Setlow, 2006), these mechanisms allow vegetative cell to undergo metabolically dormant phase, and develop tough cell structure to store microbial genetic material for their survivability. Once these endospores expose to the favorable environment, then they will germinate again. This germination of spores may be because of activation of germination receptors, either in response to nutrients called germinant (Setlow, 2003) or due to heat activation (Luu et al., 2015) or high

pressure (Setlow, 2014). Germination independent of these receptors may also be triggered by calcium chelated dipicolinic acid, dodecyl amine, or peptidoglycan fragments. However, these mechanisms may not be applicable to the food industry (Setlow, 2014). Once they germinated, they can easily multiply and grow under the favorable condition, leading to cause spoilage or pathogenicity in milk and dairy products.

2. Microbial spoilage of milk and dairy products

In general, spoilage is a term used to explain the deterioration of foods in terms of texture, color, odor or flavor to the point where it is unacceptable or unsuitable for human consumption. Microbial spoilage mainly involves the degradation of protein, carbohydrates, and fats by respective spoilage-causing microorganisms or their enzymes (Goff, 2014). Microbial spoilage of dairy products is associated with many types of bacterial species mainly spore-forming genera, psychrotrophic bacteria, coliforms, lactic acid bacteria, and fungi. A summary of different dairy products and associated typical spoilage-causing flora is shown in Table 1. These spoilage-causing microorganisms have the ability to produce deteriorative enzymes and induce textural and flavor defects in milk and dairy products. A list of milk and dairy products and their associated common microbial defects are shown in Table 2. From this spoilage-causing bacterial group, psychrotrophic microorganisms can survive and grow at or below 7°C, irrespective of their optimum growth temperature (Meer et al., 1991; Shah, 1994). Psychrotrophic bacteria are not the specific taxonomic group of microorganisms but are defined as a group of different bacterial species that can grow at 7 °C or less regardless of their

Table 1. Dairy products and associated spoilage-causing microorganisms and their activities

Milk and Milk Products	Spoilage-causing microorganisms or microbial activity
Raw milk	A wide range of spoilage-causing microorganisms, enzymatic activity, sensory-flavor associated defects
Pasteurized milk	Psychrotrophs, spore formers, enzymatic degradation, sensory-flavor associated defects
Concentrated milk	Spore formers and osmophilic fungi
Dried milk powder	Spore formers, mold, enzymatic degradation
Butter	Psychrotrophs, enzymatic degradation
Cultured buttermilk, sour cream	Psychrotrophs, coliforms, yeasts, lactic acid bacteria
Cottage cheese	Psychrotrophs, coliforms, yeasts, molds, microbial enzymatic degradation
Yogurt, yogurt-based drinks, and other fermented dairy foods	Spore formers, fungi, coliform
Cream cheese, processed cheese	Fungi, spore formers
Soft, fresh cheeses	Psychrotrophs, coliforms, fungi, lactic acid bacteria, microbial enzymatic degradation
Ripened cheeses	Fungi, lactic acid bacteria, spore-forming bacteria, microbial enzymatic degradation

(Ledenbach and Marshall, 2009)

optimal temperature of growth (IDF Bulletins, 1976). From the spoilage point of view, they are the most important spoilage-causing flora which can grow and cause spoilage in milk and dairy products, even at refrigerated temperature (Champagne et al., 1994). The common psychrotrophic bacterial species associated with raw milk including *Bacillus*, *Arthrobacter*, *Pseudomonas*, *Micrococcus*, *Aerococcus*, *Lactococcus*, *Acinetobacter*, *Alcaligenes*, *Flavobacterium*, and some of the species from *Enterobacteriaceae* family (Washam et al., 1977; Hull et al., 1992).

2.1. Spoilage-causing enzymes in milk and dairy products

Spoilage-causing bacterial species are widely prevalent to produce many diverse enzymes and induce defects especially related to texture and flavor in milk and dairy products. Furthermore, enzymes such as proteases and lipases are the most spoilage-causing enzymes that extensively influence the quality of milk and milk products by influencing aroma, texture, taste, and nutritive value (Teh et al., 2014). Many spoilage-causing bacterial species have been encountered in dairy environments such as *Pseudomonas*, *Clostridium*, *Corynebacterium*, *Streptococcus*, *Microbacterium*, *Arthrobacter*, *Acinetobacter*, *Flavobacterium* (Adams et al., 1975; Craven and Macauley, 1992; Chen et al., 2003), and the most common spore-forming genera including *Bacillus* and *Paenibacillus* species (Collins, 1981; Phillips and Griffiths, 1990; Switt et al., 2014). According to Frank (1997), the spoilage causing activity of *Bacillus* species in milk occurs primarily because of their proteolysis and lipolysis, which can even

Table 2. Milk and dairy products and associated microbial defects

Milk and Milk Products	Microbial defects
Pasteurized milk	Bitty cream, fruity flavor, gelation, sweet curdling, protease associated fouling, flat sour defect
Sterilized milk	Rancidity, off-flavor, bitterness, soapy, gelation, and sweet curdling
Cream, butter	Rancidity, off-flavor, fruity, bitterness, soapy, bitty, discoloration
Cheese	Rancidity, off-flavor, lower yield, altered coagulation time, early and late blowing defect, pink to brown discoloration, white crystal formation on surface, fruity flavor
Buttermilk, sour cream, Yogurt	Yogurt flavor (reduced diacetyl flavor), off-flavor, bitterness

(Champagne et al., 1994; Ledenbach and Marshall, 2009)

occur during refrigeration when psychrotrophic *B. cereus* is present (Montanhini and Bersot, 2012). Some of the *Bacillus* species have the ability to produce proteolytic enzymes which hydrolyze casein and cause sweet curdling of milk, and also capable of producing bitter flavor defects. In a particular study, *B. cereus* can produce phospholipase enzyme which degrades the milk fat globule membrane (MFGM) containing phospholipid, resulting in aggregation of fat (Frank, 1997). This MFGM acts as a protective layer to milk fat against a lipolytic activity and degradation of MFGM

potentially increase the susceptibility of milk fat to lipolysis. Other defects such as the development of off-flavors including bitter, fruity, rancid or flat-sour caused by the presence of a wide array of spoilage-causing microorganisms and their enzymes in pasteurized and UHT milk. Some of the proteases are thermally resistant which are potential to cause spoilage in UHT milk (Shah, 1994; Sørhaug and Stepaniak, 1997). Researchers have found that phospholipase production in raw milk can result in the development of bitter off-flavors due to the release of fatty acids (Chrisope and Marshall, 1976; Fox et al., 1976). Some of the bacterial lipases are thermally resistant which are associated with the development of rancid flavors in UHT milk (Adams and Brawley, 1981). The release of short-chain fatty acids (C4 through C8) results in the development of rancid flavor, whereas the release of long-chain fatty acids associated with a soapy flavor. Furthermore, oxidation of free unsaturated fatty acids to aldehydes and ketones, leading to cause oxidized flavor (Deeth and Fitz-Gerald, 1983), whereas fruity off-flavor is resulting from lipolysis of short-chain fatty acids, followed by esterification with alcohols (Reddy et al., 1968). Low pH in dairy products limits the lipase activity, but in some cheese varieties such as Brie and Camembert, the pH rises again to near neutrality during ripening, leading them as a more susceptible to lipolysis (Dumont et al., 1977). Dried dairy products such as whole milk powder are likely to be affected by residual heat-resistant bacterial lipases. Residual lipases in nonfat dry milk and dry whey products can hydrolyze fat in products into which they are added as ingredients (Stead, 1986).

2.2. Microbial spoilage in cheese and cultured dairy products

Cultured dairy products such as sour cream and yogurt are less prone to microbial spoilage due to the low pH of product which limits the growth of most common spoilage-causing flora proliferated in dairy environments (Rašić and Kurman, 1978; Robinson et al., 2002; Robinson et al., 2006). Most common spoilage-causing flora associated with low pH products are yeasts and molds, psychrotrophs, spore formers, coliforms which are capable of deteriorating the product quality, most likely by causing flavor and textural defects (MacBean, 2009). Common defects found in yogurt include high acid levels that produce a high acetaldehyde flavor (Vedamuthu, 1991), defects related to bitterness in a final product caused by proteolytic spore-forming bacterial species such as *B. cereus* or *B. subtilis* (Mistry, 2001). The quality of dairy products depends on the initial microflora of raw milk as the prevailed spoilage-causing bacterial species in raw milk will likely to be increased during processing. Many researchers have found that when raw milk count is $>\log 6$ cfu/mL with a higher spoilage-causing bacterial species, which could be potential to decrease the yield and quality of cheese curd (Aylward et al., 1980; Fairbairn and Law, 1987; Mohamed and Bassette, 1979; Nelson and Marshall, 1979). According to Gassem and Frank (1991), due to hydrolysis of protein especially κ -CN in raw milk by psychrotrophic bacteria, manufactured yogurt from this milk had a more-firmer gel and a higher viscosity with pronounced syneresis. Yeasts and molds are a major concern of spoilage in cultured dairy products where the low pH of the product provides a selective environment for their growth. Cousin and Marth (1977) had observed that the growth of psychrotrophs in refrigerated milk which are used for manufacturing of cottage cheese and yogurt had decreased manufacturing times and increased curd firmness. Additionally,

NCN and NPN content were observed higher for yogurt and cottage cheese made from milk precultured with psychrotrophs. Moreover, manufactured cottage cheese and yogurt were unacceptable to taste panelists. Yeasty, fermented off-flavors and gassy appearance of yogurt are often detected when yeasts grow to log 5 to log 6 cfu/g (Fleet, 1990; Rohm et al., 1992). Psychrotrophic bacterial species such as *Pseudomonas* can reduce the diacetyl content of sour cream and buttermilk which results in the development of green or yogurt-like flavor because of the reduced diacetylene compound. As similar to psychrotrophs, coliforms can also reduce the diacetyl content of sour cream and buttermilk, leading to cause flavor defects (Wang and Frank, 1981). Hogarty and Frank, (1982) observed that diacetyl lactase producing lactococci could reduce the diacetyl content during refrigerated storage (about 7°C), subsequently producing yogurt-like flavor. Yeasts can grow easily in low pH products such as sour cream and butter milk, resulting in the development of off-flavors such as fermented or yeasty favor defects. Fresh cheeses like cottage cheese, the pH of curd ranging from 4.5-4.7, whereas the pH of curd with dressing is around 5.0-5.3 which is a more favorable condition for spoilage-causing microorganisms. Furthermore, the usual salt concentration of cottage cheese with cream dressing is insufficient to limit the growth of contaminating bacterial species. In a particular study, psychrotrophic microorganisms observed to be the common spoilage-causing flora in cottage cheese which can limit the shelf life of cottage cheese by causing spoilage during refrigerated storage (Nelson and Marshall, 1977; Mohamed and Bassette, 1979; Cousin, 1982). According to Myhara and Skura (1990), bacterial spores are concentrated in cheese curd during cheese manufacturing. Therefore even one spore per mL of milk can cause spoilage in cheeses. In the case of cottage cheese, these bacterial

spores may germinate and grow during refrigerated storage and may induce spoilage related to texture or flavor in the final product. Klei et al. (1997) had evaluated the effects of somatic cells on cottage cheese yield and quality. They observed that cottage cheese (without dressing) made from milk with high somatic cells count had a lower yield efficiency. Additionally, higher proteolysis was observed during refrigerated storage in cottage cheese made from milk with high somatic cells count.

3. Research project insights and proposed objectives

As we discussed in a review, spore formers such as *Bacillus* and related genera are ubiquitously found in soil, feed (especially silage), manure, milking machines, bedding. Another source of spore formers could be biofilm formed on heat exchangers and other processing lines and equipment. This group of microorganisms can survive pasteurization and UHT treatment and find its way to the final product. Spore-forming bacteria have the ability to produce many types of spoilage-causing enzymes, namely lipases, proteinases and phospholipase which are potential to cause spoilage, mainly related to texture or flavor in the final product. Proteolysis is associated with bitterness and texture defects. The action of lipase or phospholipases on milk fat can lead to intense soapy or bitter flavor (due to short-chain fatty acids or oxidation of polyunsaturated fatty acids). In the manufacture of culture dairy products, the components such as milk protein, fat, and stabilizers are very important in the development of typical body and texture of the product during fermentation. With the current demand of clean label dairy products, some of the cultured dairy products are fermented with exopolysaccharide (EPS) producing starter cultures which can act as a natural stabilizer and eliminate the need for

added stabilizers. The presence of spore formers from the dairy processing environments during fermentation is potential to cause spoilage in cultured dairy products by degrading these components. Therefore, it is very important to characterize such spore formers present in dairy processing environments for their ability to degrade milk protein, fat, phospholipid, different types of stabilizers and EPS at their typical fermentation temperature.

In sour cream or cottage cheese manufacturing, spore-forming bacterial species could be originated from raw milk and survive the processing heat treatments or might be contaminated from biofilms formed on heat exchangers or other processing lines and equipment. Contamination of spore formers after fermentation is most likely does not influence the final product quality because of the low pH, but their contamination just prior to fermentation may bring potential changes in final product quality. During fermentation of sour cream or cottage cheese, there is sufficient time for spore formers to grow before the pH is low enough to inhibit their growth. Therefore, we hypothesize that although the number of spore formers or spores in the final fermented product may be low, and their growth during cold storage may be limited, the spoilage-causing ability of spore formers during first few hours of fermentation until the pH is low enough to inhibit their growth would cause sufficient changes in milk or sour cream mix during fermentation may influence the quality and functionality of the product. As per our knowledge, no detailed information is available on the influence of spore formers on the quality and functionality of cultured dairy products such as sour cream and cottage cheese and even, at refrigerated storage. Therefore, the objectives of this research project were:

1. To study the ability of spore formers isolated from dairy environments to degrade milk protein, fat, phospholipids, common stabilizers, and exopolysaccharides
2. To study the influence of spore formers on rheology, texture, physicochemical, sensory, and microstructure of sour cream
3. To study the influence of proteolytic spore formers on cottage cheese yield, curd size distribution, physicochemical, textural, and sensory properties

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CHAPTER 2

The ability of Spore Formers to Degrade Milk Proteins, Fat, Phospholipids, Common Stabilizers, and Exopolysaccharides

INTRODUCTION

Spore formers are prevalent in dairy environments and observed as the common spoilage-causing microflora in milk and dairy products. Their spore-forming ability increases their survivability during heat treatment which leads to their presence in a final product. At the dairy processing facilities, many sources including raw milk, biofilms formed on heat exchangers, processing lines and equipment, and finished product such as milk powder when used as an ingredient are served as the potential sources of contamination of spores and spore formers in dairy products. Mikolajcik and Simon (1978) reported that raw milk is a common source of spore formers and their counts sometimes spike beyond log 5/mL in raw milk before pasteurization. Austin and Bergeron (1995) evaluated an adherence of bacteria and biofilms formation on dairy processing equipment and reported that the formation of biofilms on a surface of dairy processing equipment is a potential source of post-processing contamination in dairy products. Stadhouders et al. (1982) also observed that biofilms formed in a milk powder plant could serve as the potential sources of bacterial contamination and subsequently, use of that contaminated milk powders in other dairy products may deteriorate the final product quality.

Most common spore-forming species found in dairy environments belong to the genus *Bacillus* and related genera including *Geobacillus*, *Paenibacillus*, etc. These

microorganisms can produce many diverse enzymes that can impact the texture of dairy products and produce typical off-flavors (Lücking et al., 2013). For example, the spoilage-causing activity of *Bacillus* in milk is primarily due to their proteolytic and lipolytic activities which can occur during refrigeration when psychrotrophic *Bacillus* spp. such as *B. cereus* are present (Frank, 1997; Montanhini and Bersot, 2012). The proteolytic activity of *B. cereus* can hydrolyze casein, which causes sweet curdling of milk (Heyndrickx and Scheldeman, 2002; Coorevits et al., 2010). According to Mistry (2001), the proteolytic activity of *B. cereus* or *B. subtilis* can develop bitterness in yogurt. Some *Bacillus* spp. such as *B. cereus*, have been reported to produce a phospholipase enzyme, which degrades the phospholipids of the milk fat globule membrane (MFGM), resulting in aggregation of fat (Frank, 1997). This MFGM acts as a protective layer to milk fat against a lipolytic activity and degradation of MFGM potentially increase the susceptibility of milk fat to lipolysis. Researchers have reported that activity of phospholipase in raw milk can result in the development of bitter off-flavors due to the release of fatty acids (Chrisope and Marshall, 1976; Fox et al., 1976). In a particular study, production of bacterial lipase in UHT milk resulted in the development of rancid flavor (Adams and Brawley, 1981).

A common thermophilic spore former, *Geobacillus* is widely accepted as the contaminant where elevated temperatures were used in manufacturing processes such as milk powder manufacturing. Some of the strains of *Geobacillus* spp. can survive pasteurization temperature during milk processing and subsequently, their spores adhere to the surface and germinate to form biofilms (Murphy et al., 1999). In a particular study, a survey of thermophilic *Bacilli* in milk powders from different countries revealed that *G.*

stearothermophilus is one of the proven problematic *Geobacillus* spp. in milk powders across the world (Rückert et al., 2004). Another common spore former present in dairy environments, such as *Paenibacillus* spp. are characterized as psychrotolerant, which can grow at refrigeration temperature (Ivy et al., 2012). In a particular study, *P. polymyxa* was identified as a primary spoilage-causing microorganism in Swedish and Norwegian refrigerated pasteurized milk (Ternström et al., 1993).

Cultured dairy products such as sour cream and yogurt, components such as milk proteins, fat, and stabilizers are essential during fermentation to develop a typical body and texture of the product. With the current demand for clean label dairy products, some of the cultured dairy products are fermented with exopolysaccharides (EPS) producing starter cultures. EPS produced during fermentation can eliminate the need for added stabilizers. Any influence on these components by spore-forming strains present in dairy environments during their fermentation may affect the final product quality by causing textural or flavor defects. Therefore, it is important to study the ability of the common spore formers present in dairy environments to degrade milk proteins, fat, phospholipids, stabilizers, and EPS at their typical fermentation temperatures. The present work was aimed to test the ability of isolated spore-forming strains from dairy environments for their spoilage-causing activities including proteolysis, lipolysis, ability to degrade phospholipids, common stabilizers (gelatin, starch, xanthan gum, and pectin), and EPS at typical sour cream (24°C) and yogurt (42°C) fermentation temperatures.

MATERIALS AND METHODS

Identification of Spore Formers

A total of 25 spore-forming strains were isolated from dairy environments including raw milk, pasteurizer balance tank, biofilms formed on heat exchangers and dry dairy ingredients including milk powder. These strains were identified to their species level using 16S rRNA gene sequence analysis which included a total of 12 types of different species such as *P. xylanilyticus*, *B. licheniformis*, *P. polymyxa*, *B. amyloliquefaciens*, *B. pumilus*, *G. stearothermophilus*, *B. mojavensis*, *B. subtilis*, *B. marisflavi*, *B. cereus*, *B. mycoides*, and *B. coagulans*.

The original isolates were grown in tryptone soya broth (Oxoid, Fisher Scientific, Pittsburgh, PA, USA) and incubated at 37°C, except *Paenibacillus* spp. and *Geobacillus* sp., which were grown at 30°C and 55°C respectively. Isolates were preserved for future use as described by Perry (1995). The actively growing isolates were pelleted out by centrifugation (Benchtop Refrigerated Centrifuge, Model: CR 412, Jouan In., VA, USA) at 3000 rpm for 10 min. Cell pellets were suspended in 0.01 M phosphate-buffered saline (PBS; Fisher Scientific, Pittsburgh, PA, USA) at pH 7.4 and preserved in 1.8 mL glycerol cryo-vials (Crobank, Copan diagnostic Inc., CA, USA) containing sterile beads. All isolates cryo-vials were stored at -75°C in a deep freezer (Ultralow Freezer, NUAIRE Inc., MN, USA).

For the identification of isolated strains as the spore former, bacterial strains were sporulated as per method suggested by Novak et al. (2005) and Martinez et al. (2017) with some modification. A sterile bead of each isolate was added to 10 mL of tryptone soya broth and incubated at 30°C for *Paenibacillus* spp., 37°C for *Bacillus* spp., and 55°C

for *Geobacillus* sp. up to 7 days and checked for the presence of spores during incubation by spore staining method (Hamouda et al., 2002). Isolates were sporulated during incubation which confirmed that isolated strains are the spore former.

Testing Spore Former for Their Spoilage-causing Activities

For the activation of spore formers, a sterile bead of each isolated strains was transferred into tryptone soya broth and incubated overnight at their respective incubation temperature. This culture activation step was performed three times by inoculating 0.1% (w/w) inoculum of a respective strain. At the third step of activation, cells at the mid-exponential phase of each isolate were pelleted out by centrifugation at 3000 rpm for 10 min. The pellets were suspended into PBS and cells were washed by vortexing for 5 min, followed by centrifugation (Benchtop Refrigerated Centrifuge, Model: CR 412, Jouan In., VA, USA) at 3000 rpm for 10 min. Washed cells pellets of isolates were suspended again in PBS and used them as a working culture. Streak plate method was used to test proteolytic and lipolytic activities, and degradation of phospholipids, and common stabilizers by streaking spore-forming strains from their working culture on pre-poured agar plates and incubated at typical sour cream (24°C) and yogurt fermentation (42°C) temperature respectively for 48 h.

Proteolytic Activity. Skim milk agar was used to identify the proteolytic activity of spore formers as described by Jones et al., (2007). A dispersion of 10% (w/v) NDM and tryptone soy agar (Oxoid, Fisher Scientific, Pittsburgh, PA, USA) were autoclaved separately at 121°C for 20 min, followed by cooling to 55°C, and mixed to a final concentration of 1% (w/v) NDM in tryptone soy agar. The presence of a clear zone on a

skim milk agar plate by a spore-forming strain indicated the proteolytic activity. An example of proteolytic spore-forming strain is shown in Fig. 1A.

Lipolytic Activity. Lipolysis was tested by streaking spore-forming strains on a spirit blue agar (Sigma-Aldrich Company Ltd., St. Lois, MO, USA). Spirit blue agar (32.15 g) was added in 1 L of distilled water and boiled to dissolve it completely, followed by sterilization at 121°C for 20 min. A lipase substrate was prepared by mixing 1 mL of Tween 80 (Fisher Scientific, Pittsburgh, PA, USA) in 400 mL of warm distilled water for 10 min, followed by vigorous mixing with 100 g of milk fat. As shown in Fig. 1B, a deep blue color around a spore-forming strain on a spirit blue agar indicated lipolytic activity (Abdou, 2003).

Phospholipids Degrading Activity. Phospholipase producing spore-forming strains was determined using a lecithin agar method developed by Chrisope et al. (1976). Lecithin agar was prepared in three fractions as follows. Fraction-A contained 3.0 g of soy lecithin (Fisher Scientific, Pittsburgh, PA, USA) and 45 ml of distilled water. Fraction-B contained 1.0 g of tryptone, 0.5 g of yeast extract, 0.5 g of glucose, 1.8 g of bacteriological agar (Oxoid, Fisher Scientific, Pittsburgh, PA, USA) and 50 ml of distilled water. Fraction-C contained 5 ml of 0.1 M CaCl₂. Fraction-A was brought to boiling and mixed homogenously. All prepared fractions were autoclaved separately at 121°C for 20 min. After cooling to 55°C, all autoclaved fractions were combined with gentle mixing. To prevent separation, plates were poured immediately with 20 ml of the mixture to prevent separation, solidified and stored for further use. In this method, phospholipase producing strain developed a turbid zone on a lecithin agar indicating phospholipids degrading ability. An example plate is shown in Fig. 1C.

Common Stabilizers Degrading Activity

Gelatin Degrading Activity. Gelatin agar was prepared to identify gelatin degrading spore-forming strain as per described by Smith and Goodner (1958). For the preparation of gelatin agar, gelatin (MP Biomedicals, Solon, OH, USA) was added at the rate of 20 g/L to the medium contains 4 g/L neopeptone, 1 g/L yeast extract, and 15 g/L Oxoid™ agar bacteriological (Fisher Scientific, Pittsburgh, PA, USA). In this test, spore-forming strain induced hydrolysis of gelatin resulted in a halo around the growth on a gelatin agar. An example plate is shown in Fig. 2A.

Starch, Xanthan Gum, and Pectin Degrading Activity. A basic medium agar (BMA) was used to identify starch, xanthan gum and pectin degrading activities of isolates. For the preparation of BMA, stabilizers (starch, xanthan gum, and pectin; MP Biomedicals Solon, OH, USA) were added separately at the rate of 2 g/L in BMA contains 2 g/L $(\text{NH}_4)_2\text{SO}_4$, 100 mg/L KCL, 500 mg/L K_2HPO_4 , 500 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 15 g/L bacteriological agar. Each stabilizer was dissolved in deionized water separately and mixed with the rest of the ingredients of BMA, followed by sterilization at 121°C for 20 min (Shawky Gebreil, 2011). Tested BMA agar plates were treated with an iodine solution [0.005% (w/v) of I_2 and 0.05% (w/v) of KI; Fisher Scientific, Pittsburgh, PA, USA]. Strains that degraded the stabilizers had developed a bright zone under a light source on BMA. Example plates of BMA containing starch, xanthan gum or pectin are shown in Fig. 2B, 2C, and 2D respectively. When BMA plate was treated with an iodine solution, it reacted with the complex polysaccharide structure of the stabilizer and observed a bright zone in areas where the stabilizer was hydrolyzed on a plate under a light source.

Quantification of Proteolytic Spore Formers for Their Level of Proteolysis

A total number of 16 and 21 spore-forming strains that were identified as being proteolytic using a skim milk agar plating method (Table 1 and 2) were subsequently, evaluated for the level of proteolysis using non-casein nitrogen (NCN) analysis and SDS-PAGE. In this test, a dispersion of NDM (11%, w/v) was heat treated to 90°C for 10 min, followed by cooling rapidly. Each proteolytic spore-forming strain was inoculated at the level of 10^3 cfu/mL from the exponential phase in NDM and incubated at 24°C and 42°C respectively for 24 h. A sample of the same NDM without added any proteolytic spore-forming strain served as a control. After incubation, all proteolytic milk samples were analyzed for NCN content as described by Zhang and Metzger (2011). Results were expressed as % NCN content of proteolyzed milk for each respective proteolytic strain used in this study.

The protein profile of same proteolyzed milk samples was determined using SDS-PAGE under a reducing condition, and subsequently, their band intensity of individual casein fractions was quantified on SDS-PAGE electrophoretogram using a Scan Wizard Bio-software (Microtek International Inc., Hsinchu, Taiwan) as per method described by Meletharayil et al. (2015). The band intensity of individual protein fractions on SDS-PAGE electrophoretogram is proportional to the amount of protein present in that particular band. Due to hydrolysis of protein by a proteolytic strain ultimately reduces the band intensity of that specific hydrolyzed protein fraction and the degree of reduction of the band intensity of individual protein would be proportional to the level of proteolysis.

In this study, the level of proteolysis was calculated for each proteolytic strain indicated as % hydrolysis by quantifying the band intensity of individual casein fractions

of proteolyzed milk using a Scan Wizard Bio-software (Microtek International Inc., Hsinchu, Taiwan) and compared it to non-proteolyzed control milk (without added any proteolytic strain). Results were demonstrated as % hydrolysis of individual casein fractions for each proteolytic strain using the following equation:

$$\% \text{ hydrolysis of individual casein fractions} = \frac{A-B}{A} \times 100$$

Where,

A = band intensity of individual casein fractions of control milk on SDS-PAGE electrophoretogram

B = band intensity of individual casein fractions of proteolyzed milk inoculated with a respective proteolytic strain on SDS-PAGE electrophoretogram

The example SDS-PAGE electrophoretogram is shown in Fig. 3.

Susceptibility of Exopolysaccharides to the Degradation

Exopolysaccharide (EPS) is considered to be a complex molecule, differs in structure, composition, molecular mass, yield, functionalities, linkage bonds, which also vary from microorganism to microorganism (Nwodo et al., 2012). Ganzle and Schwab (2005) reported that lactic acid bacteria could produce different types of EPS such as homo- and hetero- types. Some lactic acid bacteria can produce a capsular EPS, which is attached to the bacterial cell surface and a slimy EPS which is secreted in an outer environment (De Vuyst and Degeest, 1999). Because of the heterogeneity of EPS produced from microorganism to microorganism, lengthy complex processes of its isolation and the lower yield after its isolation and purification, there isn't any specific method that has been developed to identify the susceptibility of EPS for the degradation.

Therefore, an effort was made to develop a method based on the viscometric analysis to identify the susceptibility of EPS to the degradation. We hypothesized that EPS-producing starter culture would utilize lactose from the medium and produce EPS during fermentation, which increases the viscosity of the medium, whereas EPS-degrading bacterial strain will hydrolyze EPS produced during fermentation, which results in the reduction of viscosity of the medium.

Whey protein concentrate containing 30% protein, 50% lactose, 0.5% fat and 8% ash was used as a fermenting medium [10% (w/v); Daritek™, Foremost Farms, Baraboo, WI, USA], and heat treated to 90°C for 5 min, followed by a rapid cooling. *B. mojavensis* BC and *P. polymyxa* SD were selected for this test based on their other polysaccharides (starch, xanthan gum, and pectin) degrading activities (Table 1 and 2). EPS-producing (high, medium, and low ropy) commercial yogurt and sour cream cultures (Chr. Hansen, Milwaukee, WI, USA) were tested for the susceptibility of EPS to the degradation against the selected spore-forming strains. Selected strains (10^3 cfu/mL separately from the exponential phase) were inoculated separately with a respective EPS-producing yogurt or sour cream culture (0.01%, w/w) to a whey medium and fermented at 42°C or 24°C respectively to a pH of 4.6 ± 0.05 . A whey medium inoculated with a respective EPS-producing yogurt or sour cream culture without added any spore former served as a control. After fermentation, a whey medium (13 ml) inoculated with a respective spore former and its control were analyzed for the viscosity using a Stress Tech Rheometer (ATS RheoSystems, Rheological instruments inc., NJ, USA) at 100 s^{-1} for 60 s with a bob and cup assembly at 10°C.

The indication of the susceptibility of EPS to the degradation was determined by measuring the viscosity of a whey medium inoculated with EPS-producing yogurt or sour cream culture and a respective spore-forming strain to its control whey medium (inoculated with a same EPS-producing yogurt or sour cream culture but without added any spore-forming strain). The lower viscosity of a whey medium indicates a higher susceptibility of EPS to the degradation.

Statistical Analysis

Results of the susceptibility of exopolysaccharide to the degradation in terms of viscosity were analyzed by ANOVA using R-3.4.0 software (R Core Team, Vienna, Austria) and significant results were indicated by $P < 0.05$. LSD test was used to determine the significant difference between the means.

RESULTS AND DISCUSSION

Testing Spore Formers for Their Ability to the Degradation at Sour Cream and Yogurt Fermentation Temperatures

The ability of spore formers for their spoilage-causing activities such as proteolytic and lipolytic activities, degradation of phospholipids and stabilizers including gelatin, starch, xanthan gum, and pectin at sour cream (24°C) and yogurt (42°C) fermentation temperatures are shown in Tables 1 and 2, respectively. From a total of 25 spore-forming strains, 16 strains had proteolytic activity, 4 strains had lipolytic activity, 10 strains had phospholipids degrading activity, 16 strains showed gelation degrading activity and 19, 17 and 6 strains degraded starch, xanthan gum, and pectin respectively at sour cream fermentation temperature, whereas at yogurt fermentation temperature, 21 strains had proteolytic activity, 5 strains had lipolytic activity, 10 strains had

phospholipids degrading activity, 21 strains showed gelation degrading activity and 24, 17 and 6 strains degraded starch, xanthan gum, and pectin respectively. The number of strains with starch degrading activity was found to be highest, whereas the number of strains with lipolytic activity was found to be lowest among all selected activities at both fermentation temperatures. The strains that were capable of hydrolyzing protein were also able to hydrolyze gelatin and consequently, resulted in similar positive results at sour cream and yogurt (42°C) fermentation temperatures.

Some of the strains of the same bacterial species from the isolates showed similar activities at sour cream fermentation temperature (Table 1), such as the strains of *B. licheniformis* including *B. licheniformis* DBC4, *B. licheniformis* DBC5, *B. licheniformis* DBC7, and *B. licheniformis* DBC8 (positive for proteolytic activity and degradation of phospholipids, gelatin, starch, and xanthan gum). Additionally, the strains of *B. mojavensis* including *B. mojavensis* BC and *B. mojavensis* DBC1 had shown similar activities (positive for proteolytic activity and degradation of selected stabilizers such as gelatin, starch, xanthan gum, and pectin). As similar to sour cream fermentation temperature, some of the strains of the same bacterial species from the isolates showed similar activities at yogurt fermentation temperature (Table 2). Such as the strains of *B. licheniformis* including *B. licheniformis* DBC4, *B. licheniformis* DBC5, *B. licheniformis* DBC7, *B. licheniformis* DBC8, and *B. licheniformis* K1 (positive for proteolytic activity and degradation of phospholipids, gelatin, starch, and xanthan gum). Additionally, the strains of *B. mojavensis* including *B. mojavensis* BC and *B. mojavensis* DBC1 had shown similar activities (positive for proteolytic activity and degradation of stabilizers including gelatin, starch, xanthan gum, and pectin). Other researchers have also observed similar

activities among the strains of the same bacterial spp. for selected tests. In a particular study, Ghani et al. (2013) had isolated a total of 5 strains of *B. licheniformis* from soil and reported that those 5 strains of *B. licheniformis* had similar activities for selected tests including proteolytic activity and degradation of starch and pectin. However, in our study, we have identified spore-forming strains up to their species level only. Therefore, it could be possible that the strains of the same bacterial spp. with similar activities observed in our study might be the same spore-forming strain.

In contrast, some of the strains of the same bacterial species from the isolates showed different characterization for selected activities at sour cream fermentation temperature (Table 1). Such as the strains of *P. polymyxa* including *P. polymyxa* DBC1 (positive for proteolytic activity and degradation of selected stabilizers including gelatin, starch, xanthan gum, and pectin) and *P. polymyxa* SD (positive for lipolytic activity and degradation of phospholipids, starch, xanthan gum, and pectin). Likewise, the strains of *B. licheniformis* including *B. licheniformis* DBC1 (positive for all activities except degradation of pectin), *B. licheniformis* DBC2 (positive results for degradation of starch and xanthan gum), *B. licheniformis* DBC3 (positive for only xanthan gum degrading activity), *B. licheniformis* DBC6 (negative for all selected activities), and *B. licheniformis* K1 (positive for proteolytic activity and degradation of phospholipid, gelatin, and starch). Additionally, the strains of *B. pumilus* including *B. pumilus* DBC1 (positive for proteolytic activity and degradation of phospholipids, gelatin, and starch), *B. pumilus* DBC2 (positive for proteolytic, lipolytic, and gelatin degrading activities), *B. pumilus* DBC3 (negative for all selected activities) and *B. pumilus* DBC4 (positive for proteolytic activity and degradation of gelatin and starch) showed different characterization. As

similar to sour cream fermentation temperature, some of the strains of the same bacterial species from the isolates showed different characterization for selected activities at yogurt fermentation temperature (Table 2). Such as the strains of *P. polymyxa* including *P. polymyxa* DBC1 (positive for proteolytic activity and degradation of selected stabilizers including gelatin, starch, xanthan gum, and pectin) and *P. polymyxa* SD (positive for all activities). Likewise, the strains of *B. licheniformis* including *B. licheniformis* DBC1 (positive for all activities except degradation of pectin), *B. licheniformis* DBC2 (positive for proteolytic and lipolytic activities, and degradation of gelatin, starch, and xanthan gum), *B. licheniformis* DBC3 (positive for proteolytic activity and degradation of gelatin, starch, and xanthan gum), and *B. licheniformis* DBC6 (positive for proteolytic activity and degradation of gelatin and starch). Additionally, the strains of *B. pumilus* including *B. pumilus* DBC1 (positive for proteolytic activity and degradation of phospholipid, gelatin, and starch), *B. pumilus* DBC2 (positive for proteolytic and lipolytic activities, and degradation of gelatin) and *B. pumilus* DBC3 (positive for starch degrading activity), and *B. pumilus* DBC4 (positive for proteolytic activity and degradation of gelatin and starch) showed different characterization. Other researchers have also reported different activities among the strains of the same bacterial species for selected tests. Lücking et al. (2013) had isolated aerobic spore formers from industrial dairy processing environments and characterized them based on selected test activities. They observed that some of the strains of the same *Bacillus* species showed positive results for selected activities including proteolytic and lipolytic activities, whereas some of them from *Bacillus* species were found to be negative.

As shown in the spider plot graph (Fig. 4), a higher number percentage of positive strains were found at yogurt fermentation temperature for activities including proteolytic (84%) and lipolytic (20%) activities, and degradation of starch (96%) and gelatin (84%) as compared to sour cream fermentation temperature [proteolytic (64%) and lipolytic (16%) activities, and degradation of starch (76%) and gelatin (64%)], whereas phospholipids (40%), xanthan gum (68%), and pectin (24%) degrading activities resulted in a similar percentage of positive strains at both fermentation temperatures. Spore formers such as *Bacillus* spp. can grow in a wide temperature range, regardless of their optimum growth temperature. Additionally, the majority of *Bacillus* spp. are mesophilic and thermophilic in nature. According to literature, mesophilic bacteria can grow optimally between 20°C to 45°C, and their optimum growth temperature is around 37°C (Todar, 2009; Slonczewski and Foster, 2013), whereas thermophilic bacteria can grow between 41°C to 122°C (Madigan and Martinko, 2005). Yogurt fermentation temperature (42°) that we have used in our study is falling in the range of the growth temperature of mesophiles and thermophiles. Therefore, both types of mesophilic and thermophilic strains from our isolates had grown at 42°C and consequently, resulted in a higher number percentage of positive strains at yogurt fermentation temperature. In contrast, only mesophilic strains from our isolates had grown at 24°C and consequently, resulted in a lower number percentage of positive strains at sour cream fermentation temperature. For example, *B. pumilus* DBC3 and *B. licheniformis* DBC6 strains showed negative results for all activities (Table 1) at sour cream fermentation temperature, but activities such as starch degrading activity were found to be positive for *B. pumilus* DBC3, and proteolytic activity and degradation of gelatin and starch were found to be positive for *B.*

licheniformis DBC6 (Table 2) at yogurt fermentation temperatures. This finding indicates that strains, *B. pumilus* DBC3 and *B. licheniformis* DBC6 are thermophiles which had grown and degraded components of selected activities at only yogurt fermentation temperature.

Evaluation of Proteolytic Spore-Forming Strains for Their Level of Proteolysis at Sour Cream and Yogurt Fermentation Temperatures

Non-casein nitrogen (NCN) content show in Fig. 5 and 6 were used to measure the level of proteolysis of casein after proteolytic spore formers identified using a skim milk agar method (Table 1 and 2) at sour cream (24°C) and yogurt (42°C) temperatures, respectively. A wide range of proteolysis was observed (NCN content: 0.078% to 0.220% at 24°C and 0.086% to 0.304% at 42°C) among proteolytic strains at both fermentation temperatures. *B. mojavensis* BC (NCN content: 0.220% at 24°C) and *B. subtilis* DBC (NCN content: 0.304% at 42°C) had shown the highest level of proteolysis, whereas the lowest level of proteolysis was observed for *B. pumilus* DBC4 (NCN content: 0.078% at 24°C), and *B. licheniformis* DBC4 (NCN content: 0.086% at 42°C). Other proteolytic strains such as *B. cereus* DBC (NCN content: 0.161%), *B. subtilis* DBC (NCN content: 0.145%), *B. mojavensis* DBC1 (NCN content: 0.142%), and *P. polymyxa* DBC1 (NCN content: 0.131%) resulted in a higher level of proteolysis at sour cream fermentation temperature (24°C), whereas at yogurt fermentation temperature (42°C), proteolytic strains including *B. mojavensis* BC (NCN content: 0.284%), *B. mojavensis* DBC1 (NCN content: 0.272%), *B. cereus* DBC (NCN content: 0.244%), and *B. licheniformis* DBC6 (NCN content: 0.200%) showed a higher level of proteolysis.

The percentage hydrolysis of individual casein fractions was calculated for each proteolytic strain from their SDS-PAGE electrophoretograms at sour cream (24°C) and yogurt (42°C) fermentation temperatures shown in Tables 3 and 4, respectively. At sour cream fermentation temperature, proteolytic strains such as *B. subtilis* DBC, *B. cereus* DBC, *P. polymyxa* DBC1, *B. mojavensis* BC, and *B. mojavensis* DBC1 had hydrolyzed κ -CN entirely (indicated as ND in Table 3). Additionally, the same proteolytic strains were observed with higher percentage hydrolysis of β -CN. Same proteolytic strains were also found with a higher percentage of NCN content (Fig. 5). The strains of *B. mojavensis* including *B. mojavensis* BC and *B. mojavensis* DBC1 were capable of hydrolysis α S₂- and α S₁- CN at a higher level. At yogurt fermentation temperature, all proteolytic strains had hydrolyzed κ -CN entirely (indicated as ND in Table 4). Proteolytic strains such as *B. subtilis* DBC, *B. cereus* DBC, *B. mojavensis* BC, *B. mojavensis* DBC1, and *B. licheniformis* DBC6 were observed with higher percentage hydrolysis of all individual casein fractions. Same proteolytic strains were also found with a higher percentage of NCN content (Fig. 6). Strongin and Stepanov (1981) reported that *Bacillus* species have more extracellular and intracellular proteolytic activity as compared to any other bacterial species. Additionally, casein is more susceptible than whey protein to bacterial proteinases (Frank, 2007), and among casein fractions, β - and κ - CN are more sensitive to bacterial proteinases than α s-CN (Gebre-Egziabher et al., 1980). In our study, we observed a similar trend with higher percentage hydrolysis of β - and κ - CN by proteolytic strains. Due to the capability of degrading casein, especially β - and κ -CN, these proteolytic strains are potential to cause texture or flavor defects in sour cream and yogurt. Moreover, hydrolysis β -CN is primarily associated with the development of

bitterness. Fernandez-Garcia (1994) reported that cheeses containing the *B. subtilis* protease developed bitterness, and sticky and crumbly texture because of the intense breakdown of β -CN.

The example of high (lane b) and low (lane c) proteolytic strains with a control (lane a) are shown on SDS-PAGE electrophoretogram in Fig. 7A. The extensive proteolysis of milk by highly proteolytic strains resulted in a curdling of milk, the example highly proteolytic strains, *B. mojavensis* BC (tube q) at 24°C and *B. subtilis* DBC (tube r) at 42°C are shown in Fig. 7B with a control (tube p). This is due to the extensive hydrolysis of casein, especially κ -CN which resulted in a steric destabilization of casein micelles and consequently, caused a curdling of milk. Several studies have reported that the proteolytic activity of *B. cereus* resulted in a sweet curdling defect in milk (Frank, 1997; Heyndrickx and Scheldeman, 2002; Coorevits et al., 2010).

Evaluation of the Susceptibility of Exopolysaccharide to the Degradation by Spore Formers

As shown in Fig. 8, the viscosity of a control whey medium (inoculated with a respective EPS-producing yogurt or sour cream culture without added any spore former) is indicated as a treatment C, whereas the viscosity of whey medium inoculated with a respective spore formers is indicated as a treatment T1 for *B. mojavensis* BC strain and T2 for *P. polymyxa* SD strain.

A higher ($P < 0.05$) reduction in viscosity was observed in the case of *B. mojavensis* BC (T1) strain added whey medium for all EPS-producing yogurt and sour cream cultures as compared to *P. polymyxa* SD strain (T2). This indicates that the susceptibility of EPS to the degradation was higher by *B. mojavensis* BC strain for all

EPS-producing yogurt and sour cream cultures as compared to *P. polymyxa* SD strain. In a previous section, *B. mojavensis* BC was identified as a proteolytic strain with a higher level of proteolysis (Fig. 5 and 6). Additionally, *B. mojavensis* BC had degraded other polysaccharides including starch, xanthan gum, and pectin (Table 1 and 2). These observations indicate that *B. mojavensis* BC strain is capable of producing many diverse enzymes that could have degraded EPS at a higher level as compared to *P. polymyxa* SD. The level of susceptibility of EPS to the degradation was found to be different among all EPS-producing yogurt and sour cream cultures used in this study. The variability in susceptibility of EPS to the degradation could be due to the diversity of structure, composition, molecular mass, yield, and functionalities. For example, the susceptibility of EPS was found to be highest in low ropy EPS-producing yogurt culture (viscosity of C: 22.4 cP, T1: 12.0 cP and T2: 15.1 cP) as compared to a high (viscosity of C: 30.3 cP, T1: 23.2 cP and T2: 25.8 cP) ropy EPS-producing yogurt culture by selected spore formers. This is due to EPS produced from a low ropy EPS-producing yogurt culture that could be structurally or compositionally weaker, or that the less ropy behavior of EPS in a whey medium could have exposed more area of EPS to hydrolysis, whereas EPS produced from a high ropy EPS-producing yogurt culture that could be structurally or compositionally stronger or that might have restricted the access of degrading enzymes to the EPS due to its high ropiness.

CONCLUSION

The ability of spore formers to the degradation for selected components in this study was higher at a yogurt fermentation temperature (42°C) as compared to sour cream fermentation temperature (24°C). The order of susceptibility to the degradation for tested

strains was starch > xanthan gum > protein = gelatin > phospholipid > pectin > fat at 24°C, and starch > protein = gelatin > xanthan gum > phospholipids > pectin > fat at 42°C. Proteolytic strains with a higher level of proteolysis degraded casein, primarily β - and κ - CN, which can cause texture or flavor defects in sour cream and yogurt. EPS produced by a low ropy EPS-producing yogurt culture was more susceptible to the degradation by selected spore formers. The findings of this study should be of interest to the dairy industry because of the prevalence of stabilizers and EPS-producing cultures used in sour cream and yogurt. Our observations indicate that the presence of spore-forming bacteria found in dairy environments may negatively impact the texture or flavor of fermented dairy products.

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Table 1. Testing spore-forming strains for their spoilage-causing activities at sour cream fermentation temperature (24°C)

	Proteolytic activity	Lipolytic activity	Phospholipids degrading activity	Gelatin degrading activity	Starch degrading activity	Xanthan gum degrading activity	Pectin degrading activity
Spore formers							
<i>P. xylanilyticus</i> DBC	-	+	-	-	+	+	-
<i>P. polymyxa</i> DBC1	+	-	-	+	+	+	+
<i>P. polymyxa</i> SD	-	+	+	-	+	+	+
<i>B. pumilus</i> DBC1	+	-	+	+	+	-	-
<i>B. pumilus</i> DBC2	+	+	-	+	-	-	-
<i>B. pumilus</i> DBC3	-	-	-	-	-	-	-
<i>B. pumilus</i> DBC4	+	-	-	+	+	-	-
<i>B. licheniformis</i> DBC1	+	+	+	+	+	+	-
<i>B. licheniformis</i> DBC2	-	-	-	-	+	+	-
<i>B. licheniformis</i> DBC3	-	-	-	-	-	+	-
<i>B. licheniformis</i> DBC4	+	-	+	+	+	+	-
<i>B. licheniformis</i> DBC5	+	-	+	+	+	+	-
<i>B. licheniformis</i> DBC6	-	-	-	-	-	-	-
<i>B. licheniformis</i> DBC7	+	-	+	+	+	+	-
<i>B. licheniformis</i> DBC8	+	-	+	+	+	+	-
<i>B. licheniformis</i> K1	+	-	+	+	+	-	-
<i>B. mojavensis</i> BC	+	-	-	+	+	+	+
<i>B. mojavensis</i> DBC1	+	-	-	+	+	+	+
<i>B. marisflavi</i> DBC	-	-	-	-	-	+	-
<i>B. amyloliquefaciens</i> DBC	+	-	-	+	+	+	+
<i>G. stearothermophilus</i> DBC1	-	-	-	-	-	+	-
<i>B. cereus</i> DBC	+	-	+	+	+	+	-
<i>B. mycoides</i> DBC	-	-	+	-	+	-	-
<i>B. coagulans</i> SD	+	-	-	+	+	-	-
<i>B. subtilis</i> DBC	+	-	-	+	+	+	+

(+) - positive result, (-) - negative result

Table 2. Testing spore-forming strains for their spoilage-causing activities at yogurt fermentation temperature (42°C)

Spore formers	Proteolytic activity	Lipolytic activity	Phospholipids degrading activity	Gelatin degrading activity	Starch degrading activity	Xanthan gum degrading activity	Pectin degrading activity
<i>P. xylanilyticus</i> DBC	-	+	-	-	+	+	-
<i>P. polymyxa</i> DBC1	+	-	-	+	+	+	+
<i>P. polymyxa</i> SD	+	+	+	+	+	+	+
<i>B. pumilus</i> DBC1	+	-	+	+	+	-	-
<i>B. pumilus</i> DBC2	+	+	-	+	-	-	-
<i>B. pumilus</i> DBC3	-	-	-	-	+	-	-
<i>B. pumilus</i> DBC4	+	-	-	+	+	-	-
<i>B. licheniformis</i> DBC1	+	+	+	+	+	+	-
<i>B. licheniformis</i> DBC2	+	+	-	+	+	+	-
<i>B. licheniformis</i> DBC3	+	-	-	+	+	+	-
<i>B. licheniformis</i> DBC4	+	-	+	+	+	+	-
<i>B. licheniformis</i> DBC5	+	-	+	+	+	+	-
<i>B. licheniformis</i> DBC6	+	-	-	+	+	-	-
<i>B. licheniformis</i> DBC7	+	-	+	+	+	+	-
<i>B. licheniformis</i> DBC8	+	-	+	+	+	+	-
<i>B. licheniformis</i> K1	+	-	+	+	+	+	-
<i>B. mojavensis</i> BC	+	-	-	+	+	+	+
<i>B. mojavensis</i> DBC1	+	-	-	+	+	+	+
<i>B. marisflavi</i> DBC	+	-	-	+	+	-	-
<i>B. amyloliquefaciens</i> DBC	+	-	-	+	+	+	+
<i>G. stearothermophilus</i> DBC1	-	-	-	-	+	+	-
<i>B. cereus</i> DBC	+	-	+	+	+	+	-
<i>B. mycoides</i> DBC	-	-	+	-	+	-	-
<i>B. coagulans</i> SD	+	-	-	+	+	-	-
<i>B. subtilis</i> DBC	+	-	-	+	+	+	+

(+) - positive result, (-) - negative result

Table 3. Percentage hydrolysis of individual casein fractions by proteolytic strains at sour cream fermentation temperature (24°C)

Proteolytic strains	% hydrolysis of individual casein fraction			
	α S ₂ -CN	α S ₁ -CN	β -CN	κ -CN
<i>B. subtilis</i> DBC	8.6	10.1	22.2	ND
<i>B. cereus</i> DBC	6.3	9.9	31.9	ND
<i>P. polymyxa</i> DBC1	4.8	13.1	22.8	ND
<i>B. amyloliquefacience</i> DBC	4.8	9.1	14.3	35.8
<i>B. coagulans</i> SD	6.3	9.9	10.3	14.5
<i>B. mojavensis</i> BC	15.8	39.0	57.6	ND
<i>B. mojavensis</i> DBC1	10.5	29.8	37.6	ND
<i>B. pumilus</i> DBC1	6.7	4.0	7.4	9.4
<i>B. pumilus</i> DBC2	2.9	4.5	7.9	10.4
<i>B. pumilus</i> DBC4	3.8	3.5	10.1	8.5
<i>B. licheniformis</i> DBC1	3.8	5.6	6.9	16.0
<i>B. licheniformis</i> DBC4	4.2	7.5	14.8	10.3
<i>B. licheniformis</i> DBC5	3.2	14.1	17.1	15.9
<i>B. licheniformis</i> DBC7	5.3	12.7	16.2	11.9
<i>B. licheniformis</i> DBC8	7.4	11.7	15.2	16.7
<i>B. licheniformis</i> K1	5.3	5.2	8.1	19.8

ND- not detected on SDS-PAGE electrophoretogram

Table 4. Percentage hydrolysis of individual casein fractions by proteolytic strains at yogurt fermentation temperature (42°C)

Proteolytic strains	% hydrolysis of individual casein fraction			
	α S ₂ -CN	α S ₁ -CN	β -CN	κ -CN
<i>B. subtilis</i> DBC	27.1	50.0	75.8	ND
<i>B. cereus</i> DBC	28.2	28.9	56.2	ND
<i>B. amyloliquefacience</i> DBC	6.0	12.2	12.6	ND
<i>B. marisflavi</i> DBC	9.4	11.3	11.9	ND
<i>B. coagulans</i> SD	8.9	14.1	15.3	ND
<i>P. polymyxa</i> DBC1	13.1	14.4	14.9	ND
<i>P. polymyxa</i> SD	10.0	12.0	14.7	ND
<i>B. mojavensis</i> DBC1	15.3	19.1	28.4	ND
<i>B. mojavensis</i> BC	12.2	23.6	39.5	ND
<i>B. pumilus</i> DBC1	8.3	11.0	12.0	ND
<i>B. pumilus</i> DBC2	6.0	12.7	15.4	ND
<i>B. pumilus</i> DBC4	8.2	10.3	16.0	ND
<i>B. licheniformis</i> DBC1	8.3	11.6	11.4	ND
<i>B. licheniformis</i> DBC2	7.1	6.6	9.7	ND
<i>B. licheniformis</i> DBC3	10.6	6.7	12.9	ND
<i>B. licheniformis</i> DBC4	9.4	12.9	13.4	ND
<i>B. licheniformis</i> DBC5	7.8	10.5	11.1	ND
<i>B. licheniformis</i> DBC6	16.7	15.7	32.6	ND
<i>B. licheniformis</i> DBC7	12.2	12.6	14.2	ND
<i>B. licheniformis</i> DBC8	6.7	9.4	10.5	ND
<i>B. licheniformis</i> K1	5.6	11.5	14.2	ND

ND- not detected on SDS-PAGE electrophoretogram

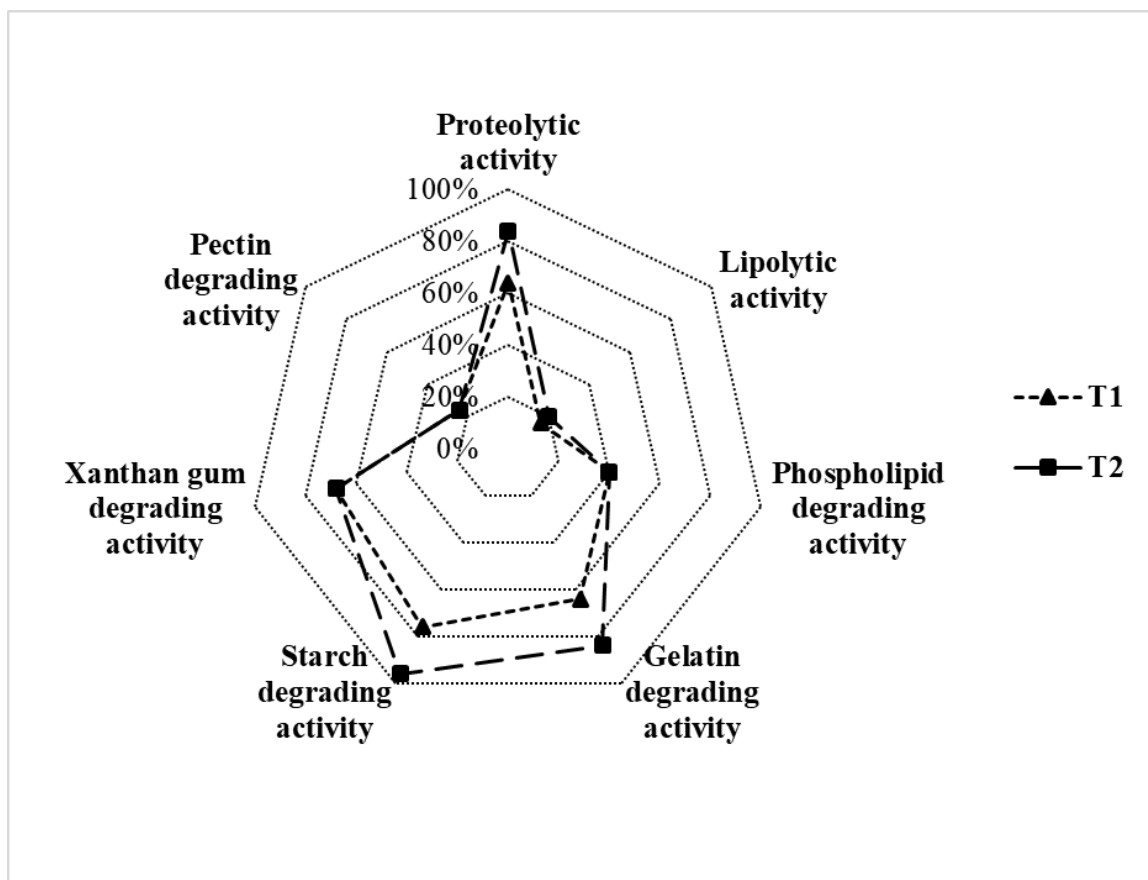


Figure 1. Percentage of positive isolates to a total of twenty-five isolates for selected spoilage-causing activities including proteolytic and lipolytic activities, and degradation of phospholipid and stabilizers such as gelatin, starch, xanthan gum, and pectin at T1) sour cream fermentation temperature (24°C), and T2) yogurt fermentation temperature (42°C)

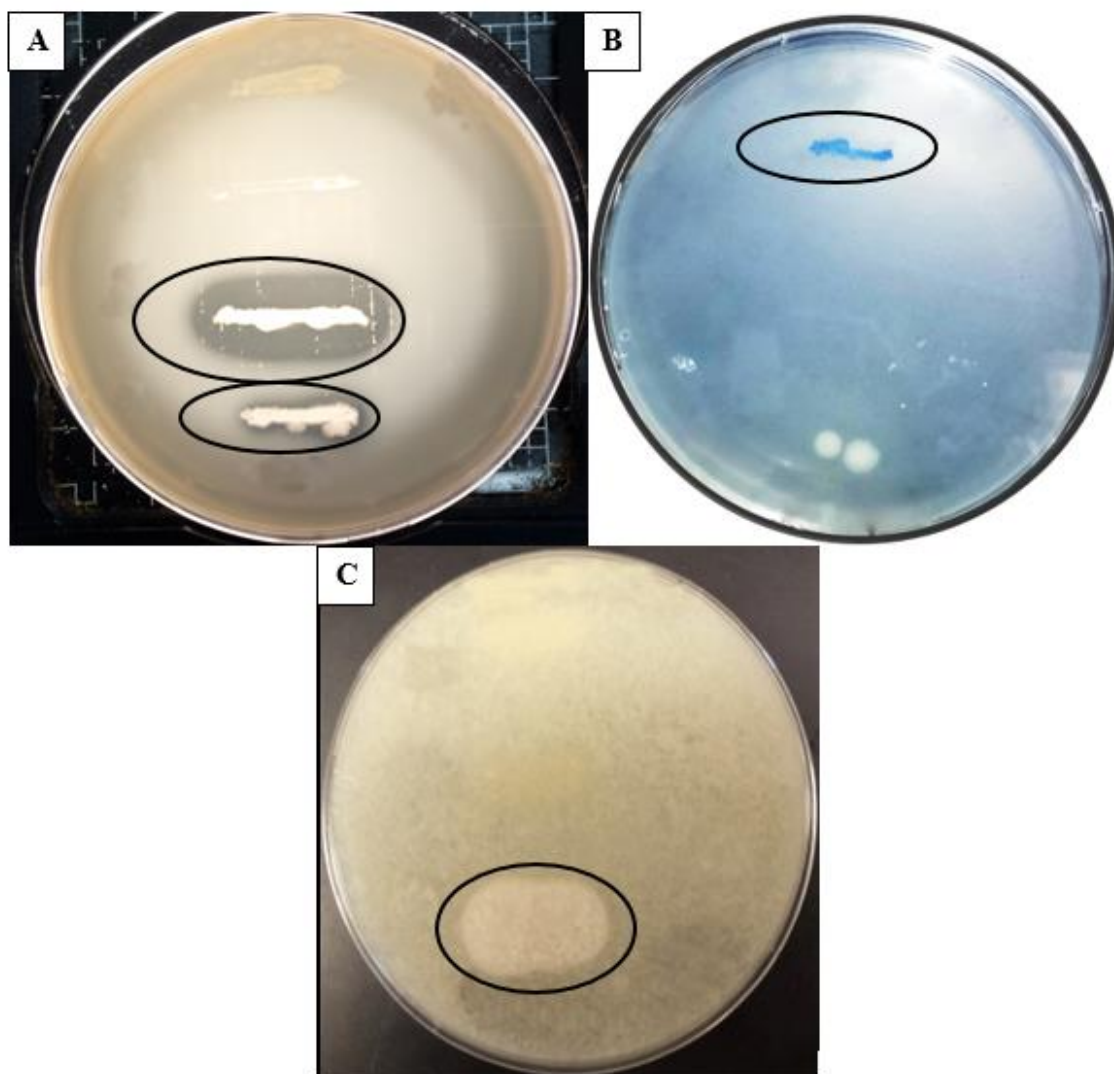


Figure 2. Isolates with A) proteolytic activity indicated as the development of clear zone on a skim milk agar, B) lipolytic activity indicated as the development of a deep blue color around the growth on a spirit blue agar, and C) phospholipid degrading activity indicated as the development of a turbid zone on a lecithin agar

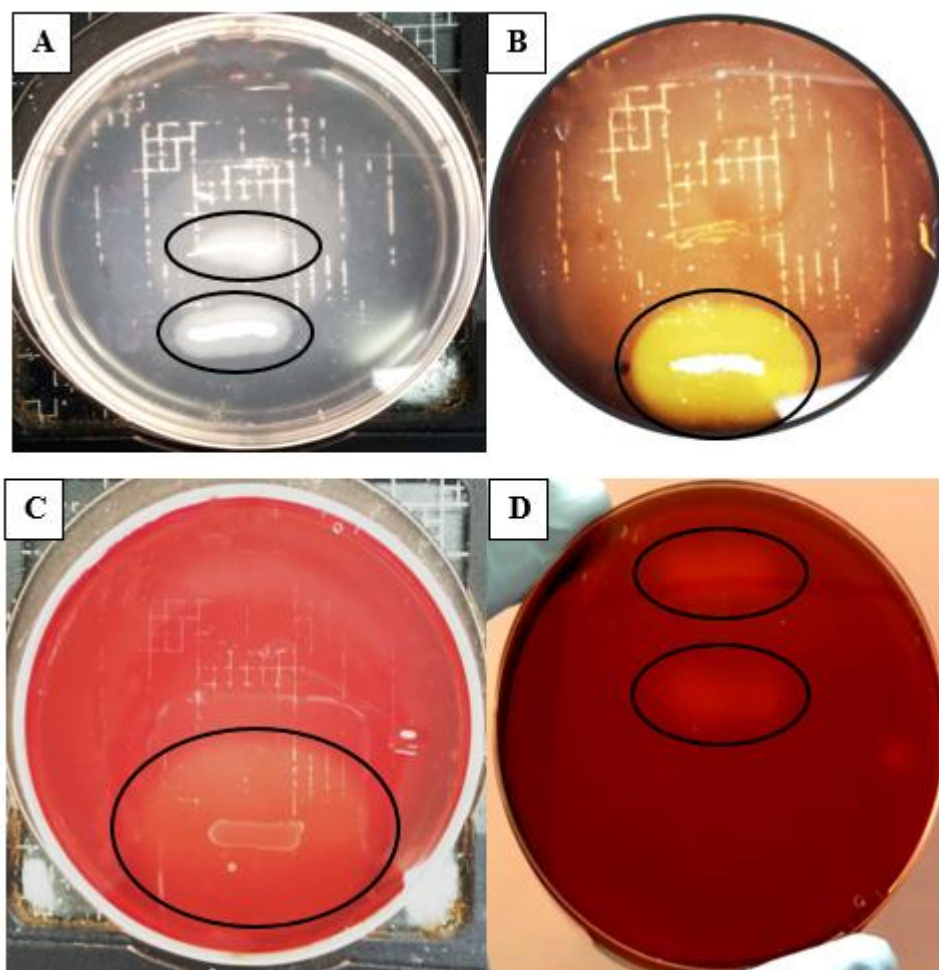


Figure 3. Isolates with stabilizers degrading activities: A) development of a halo around the growth on a gelatin agar indicated gelatin degrading activity, and development of a bright zone on a basic medium agar (BMA) contained B) starch, C) xanthan gum and, D) pectin when treated with an iodine solution indicated starch, xanthan gum and pectin degrading activities respectively

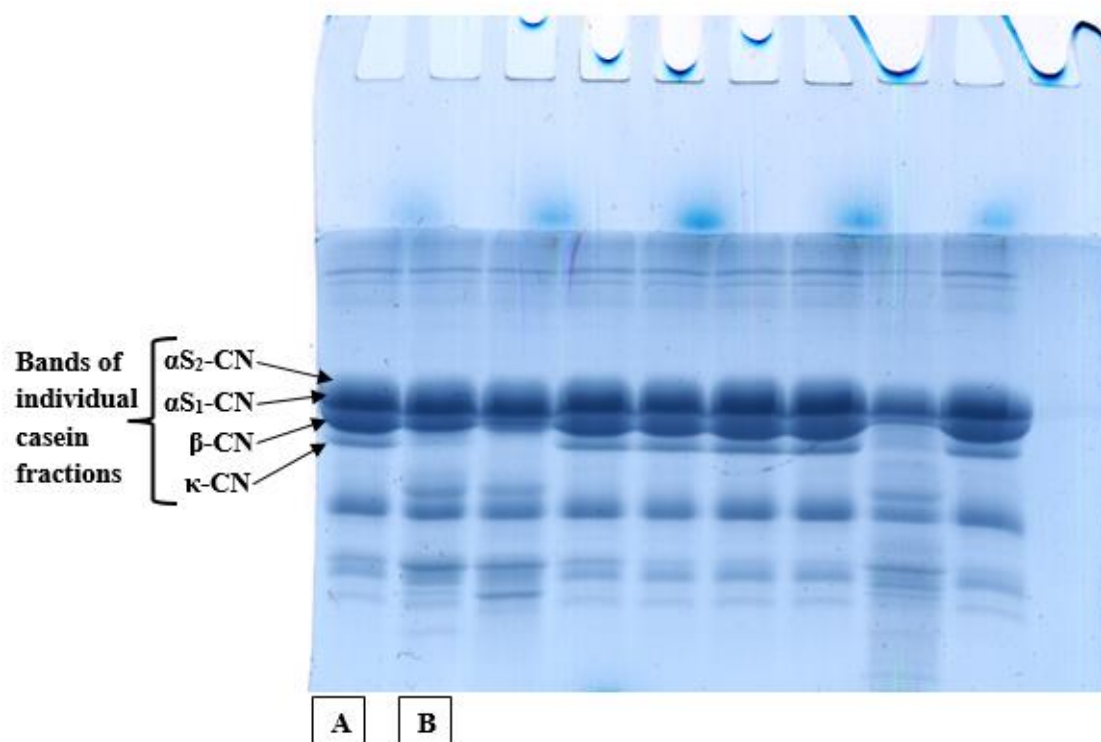


Figure 4. SDS-PAGE electrophoretogram indicating individual casein fractions of A) non-hydrolyzed control milk sample, and B) hydrolyzed milk sample by a proteolytic strain

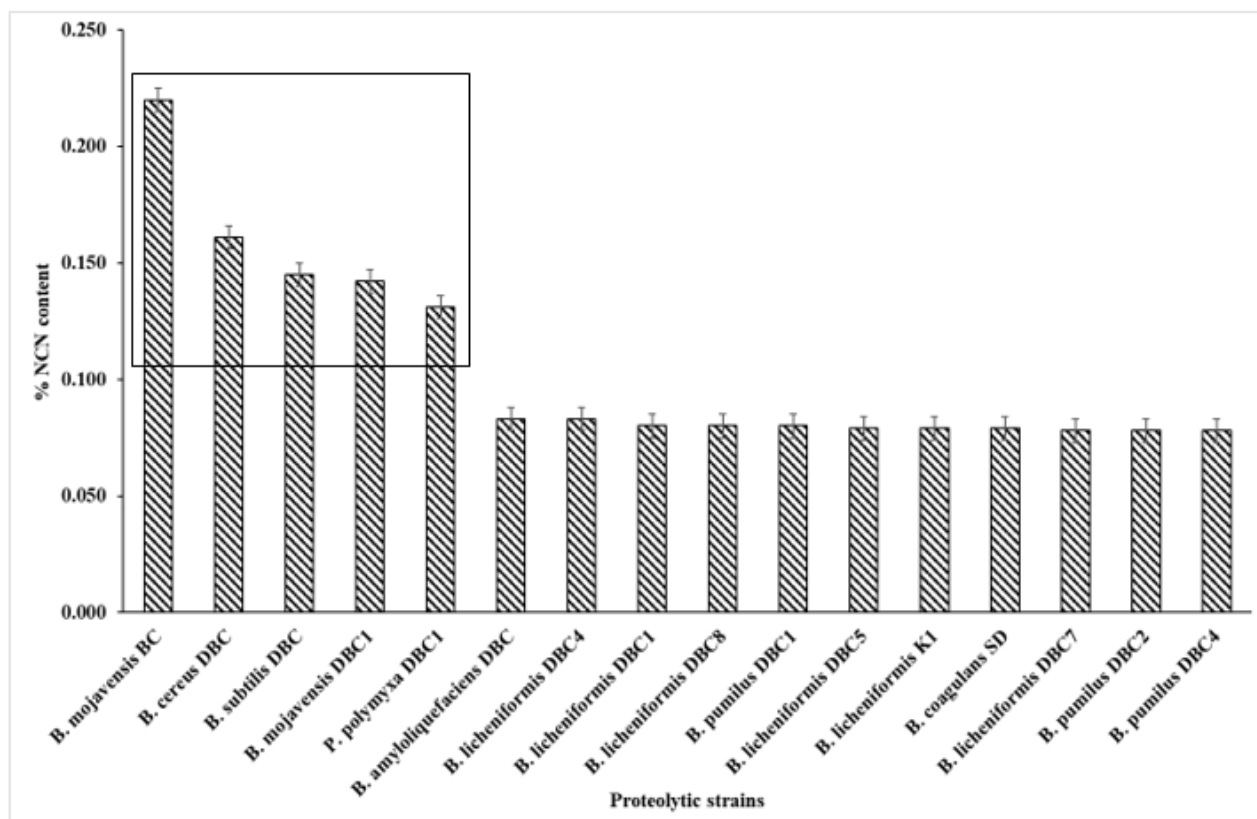


Figure 5. Percentage non-casein nitrogen (NCN) content of proteolyzed milk samples inoculated with respective proteolytic strains at sour cream fermentation temperature (24°C)

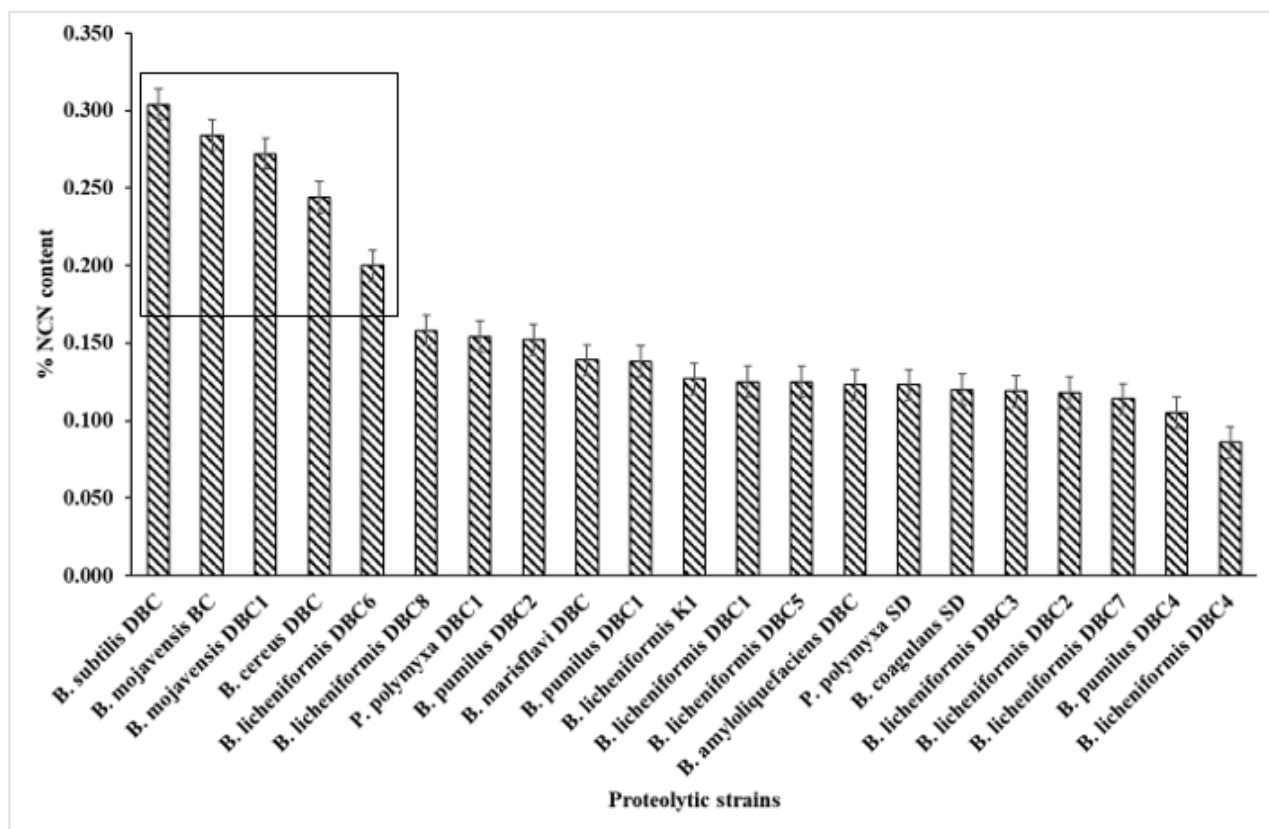


Figure 6. Percentage non-casein nitrogen (NCN) content of proteolyzed milk samples inoculated with respective proteolytic strains at yogurt fermentation temperature (42°C)

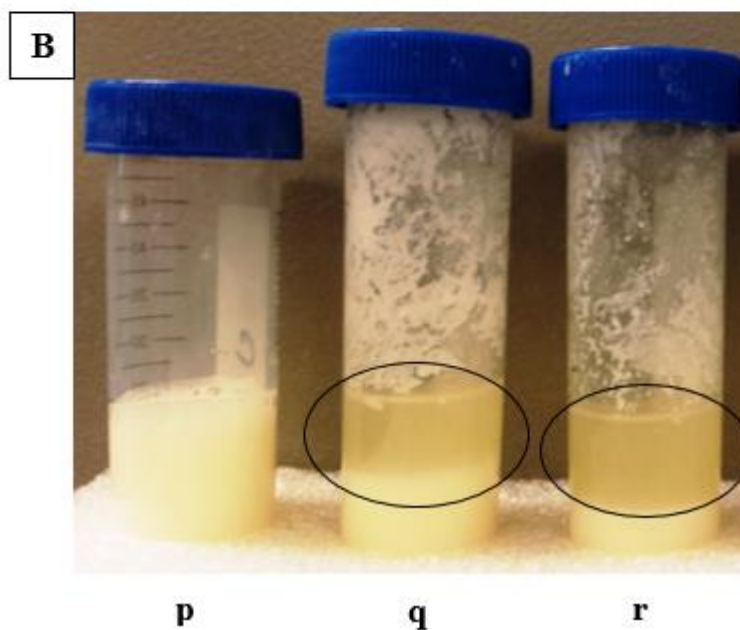
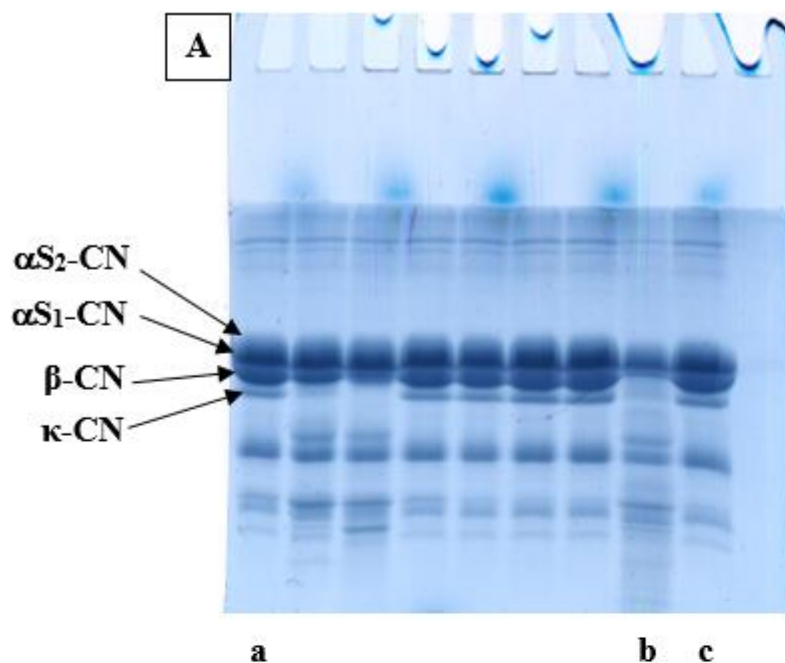


Figure 7. A) SDS-PAGE electrophoretogram indicating individual casein fractions of hydrolyzed milk by high (lane b) and low (lane c) proteolytic strains along with a non-hydrolyzed control milk sample (lane a), B) non-hydrolyzed control milk (tube p) and curdled milk caused by highly proteolytic *B. mojavensis* BC (tube q) at 24°C and *B. subtilis* DBC (tube r) at 42°C respectively after 24 h of incubation

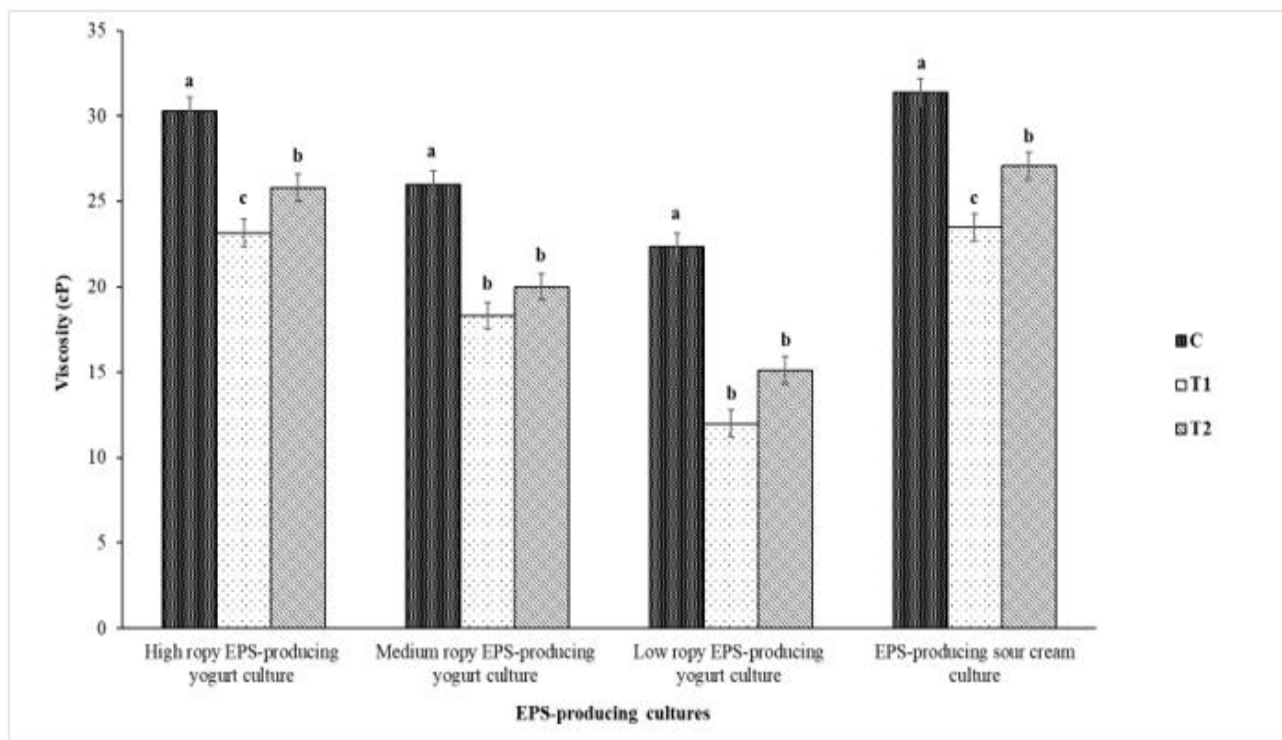


Figure 8. Viscosity of a whey medium inoculated with a low rop, medium rop and high rop EPS-producing yogurt culture or EPS-producing sour cream culture with three treatments: C) without added any spore former served as a control, T1) added with *B. mojavensis* BC, and T2) added with *P. polymyxa* SD. Different small letter within the treatments of each EPS-producing yogurt or sour cream culture indicating that results are significantly different ($P < 0.05$).

CHAPTER 3

Influence of Spore Formers on Rheology, Texture, Physicochemical, Sensory, and Microstructure of Sour Cream

INTRODUCTION

Fermentation is one of the most important steps in the manufacturing of cultured dairy products (Lucey, 2004). Any changes occur during fermentation leading to affect textural, rheological, and physicochemical properties of the final product (Lucey and Singh, 1997; Lucey, 2002). The cultured dairy products such as sour cream, milk components including protein and fat contribute to the essential role in structure formation and its functionality. Any deteriorative activity by bacterial contamination such as hydrolysis of protein or lipolysis of fat during fermentation may lead to affect sour cream properties by influencing its texture or flavor. Spore-forming microorganisms such as *Bacillus* spp. are commonly found as the contaminants in milk and dairy products. Their spore-forming ability facilitates them to survive high heat treatments during processing which leads their presence in the final product. Some of the strains of *Bacillus* spp. have the ability to develop biofilms in dairy processing environments which serve as the potential sources of contamination in dairy products. *Bacillus* spp. are widely accepted for their ability to produce many diverse enzymes, primarily proteases, and lipases which can influence the value and quality of dairy products by affecting their sensory properties including texture, taste, aroma, and nutritional value (Teh et al., 2014). Researchers have observed that the proteolytic activity of *Bacillus* is associated with texture or flavor defects such as bitterness, whereas the lipolytic activity is responsible

for rancidity or aggregation of fat (Davies and Wilkinson, 1973; Frank, 1997). In a particular study, such enzymatic activities of *B. cereus* were found to be continuous even during refrigerated storage (Frank, 1997; Montanhini and Bersot, 2012). Some of the *Bacillus* spp., for example, *B. cereus* has been reported to produce a phospholipase enzyme (Frank, 1997) which has the ability to degrade phospholipids situated in a milk fat globule membrane that leads to increasing the susceptibility of milk fat to lipolysis.

Many studies have been reported that *B. licheniformis* and *B. subtilis* are the most commonly found *Bacillus* spp. in dairy environments. According to Buehner et al. (2014), *B. licheniformis* was the predominant spp. identified in 62.4% of winter and 49.4% of summer samples, followed by *B. subtilis* with 9.4% of the remaining winter isolates. A survey on the incidence of spore-forming bacteria in dairy farms in New Zealand revealed that *B. licheniformis* was the most predominant spp. among the collected isolates across the dairy farms (Gupta and Brightwell, 2017). Many researchers have reported that in general, *B. licheniformis* and *B. subtilis* are the predominant mesophiles present in dairy environments (Sutherland and Murdoch, 1994; Tatzel et al., 1994; Lukasova et al., 2001).

As per our knowledge, there is no detailed information available on the contamination of spore formers during fermentation and their influence on sour cream properties during storage. Therefore, in this study, we have used the most common spore formers such as *B. subtilis* and *B. licheniformis* which were isolated from dairy environments and evaluated their influence on microstructure, rheology, textural, physicochemical, and sensory properties of sour cream.

MATERIALS AND METHODS

Spore formers and Sour Cream Starter Cultures

Spore-forming bacilli, *B. subtilis* DBC and *B. licheniformis* DBC1 were used in this study which was isolated from dairy environments and identified them up to their species level using the 16S rRNA gene sequencing technique. The original isolates were grown in Oxoid™ tryptone soya broth (Fisher Scientific, Pittsburgh, PA, USA) and incubated overnight at 37°C. Isolates were preserved for future use as described by Perry (1995). The actively growing isolates were pelleted out by centrifugation (Benchtop Refrigerated Centrifuge, Model: CR 412, Jouan Inc., VA, USA) at 3000 rpm for 10 min. Cell pellets were suspended in 0.01 M phosphate-buffered saline (PBS; Fisher Scientific, Pittsburgh, PA, USA) at pH 7.4 and preserved in 1.8 mL glycerol cryo-vials (Crobank™, Copan diagnostic Inc., CA, USA) containing sterile beads. All isolates cryo-vials were stored at -75°C in a deep freezer (Ultralow Freezer, NUAIRE Inc., MN, USA).

Isolated *Bacillus* spp. were identified as a spore former by sporulating them as per method suggested by Novak et al. (2005) and Martinez et al. (2017) with some modification. A sterile bead of each isolate was added to 10 mL of Oxoid™ tryptone soya broth and incubated at 37°C up to 5 days and checked for the presence of spores during incubation by spore staining method (Hamouda et al., 2002). Both *Bacillus* spp., *B. subtilis* DBC and *B. licheniformis* DBC1 were sporulated during incubation which confirmed that selected *Bacillus* spp. are spore formers.

For the activation of selected *Bacillus* spp. from frozen cryo-vials, a sterile bead of each isolate was transferred into Oxoid™ tryptone soya broth (Fisher Scientific, Pittsburgh, PA, USA) and incubated overnight at 37°C. This culture activation step was

performed three times with 0.1% (w/w) inoculation. At the third step of activation, cells at the mid-exponential phase of each isolate were pelleted out by centrifugation at 3000 rpm for 10 min. The pellets were suspended into 0.01 M phosphate-buffered saline (PBS; Fisher Scientific, Pittsburgh, PA, USA) and cells were washed by vortexing for 5 min, followed by centrifugation (Benchtop Refrigerated Centrifuge, Model: CR 412, Jouan In., VA, USA) at 3000 rpm for 10 min. Washed cells pellets of isolates were resuspended again in PBS and used them as a working culture.

Selected *Bacillus* spp., *B. subtilis* DBC as the proteolytic strain, and *B. licheniformis* DBC1 as the proteolytic, lipolytic, and phospholipids degrading strain were characterized based on a plating method such as proteolytic activity using a skim milk agar method (Jones et al., 2007), lipolytic activity using a spirit blue agar method (Abdou, 2003), and phospholipid degrading activity using a lecithin agar method (Chrisope et al., 1976). Furthermore, *Bacillus* spp. were quantified for their level of proteolysis by analyzing non-casein nitrogen (NCN) content in proteolyzed milk at sour cream fermentation temperature as described by Zhang and Metzger (2011). *B. subtilis* DBC and *B. licheniformis* DBC1 were inoculated individually approximately at the level of 10^3 cfu/mL from the exponential phase in a dispersion of heat treated non-fat dry milk (11%, w/v) and incubated at 26°C for 24 h. After incubation, selected *Bacillus* spp. were analyzed for NCN content. Results indicated that *B. subtilis* had a higher proteolytic activity (NCN content: 0.182%) as compared to *B. licheniformis* (NCN content: 0.092%). Commercial sour cream culture (Chr. Hansen, Milwaukee, WI), F DVS R-604 and F DVS DSG-HB eXact were used in 80:20 proportion as an acidifying culture for the manufacturing of sour cream.

Sour Cream Blend and Experimental Design

Heat treated sour cream blend ($18.31 \pm 0.078\%$ fat, $2.59 \pm 0.052\%$ protein, and $25.35 \pm 0.051\%$ total solids) was received from a local sour cream manufacturing plant. This blend was set to a temperature at 26°C and inoculated with sour cream cultures at 0.01% (w/w), co-cultured with d *Bacillus* spp. separately or together approximately at 10^3 cfu/g from the exponential phase, whereas sour cream blend without added any *Bacillus* was served as a control. Cultured sour cream blend was transferred into sterile plastic cups (approximately 100 g) with lids and fermented at 26°C to a $\text{pH } 4.6 \pm 0.05$, followed by cooling and storage at 4°C . Microbial counts and pH was monitored during fermentation, whereas microbial counts, rheological and textural properties, graininess, proteolysis, phospholipids and volatile free fatty acids content, and sensory properties were analyzed during storage at 4°C . In this study, the experimental block consists of a total of 4 treatments (Table 1) from the same sour cream blend. This design of experiment was replicated three times.

Microbial Counts

Starter culture count, *Bacillus* count, and pH were monitored during fermentation and on 1st, 15th, and 30th day of storage. Starter cultures colonies were enumerated using Oxoid™ M17 agar (Fisher Scientific, Pittsburgh, PA, USA) as a selective medium under anaerobic condition according to method describer by Elliker et al. (1956) and Terzaghi and Sandine (1975). M17 agar (48.25 g) was suspended in 950 mL of distilled water and brought gently to the boil, followed by sterilization at 121°C for 20 min, and cooled to 55°C . Sterile lactose solution (10%, w/v) of 50 mL was added to the autoclaved M17 agar

and used as a selective medium to enumerate starter culture colonies. For the preparation of 10% (w/v) sterile lactose solution, lactose powder (Fisher Scientific, Pittsburgh, PA, USA) of 10 g was dissolved in 100 mL of distilled water and passing it through a sterile membrane filter (0.22 μ m pore size; MilliporeSigma, Burlington, MA, USA).

Bacillus colonies were encountered on Oxoid™ M17 agar containing bromocresol green (TSABG) as a pH indicator (pK 4.6) under the aerobic condition as described by McDonald et al. (1987) and incubated at 37°C for 24 h. *Bacillus* colonies remained blue on TSABG, whereas acid producing starter cultures colonies turned out as green colonies. The preparation of dilutions for sour cream samples, plating, and counting of colonies was performed according to methods described by Laird et al. (2004) and Duncan et al., (2004).

Proteolysis Measurement

Sour cream was analyzed for non-protein nitrogen [NPN (%)] content as the indication of proteolysis described by Hooi et al. (2004) with a sample size of 5 g. NPN content of sour cream was measured on the 1st, 15th, and 30th day of storage.

The individual protein fractions present in sour cream was determined using capillary gel electrophoresis (CE) as per the method described by Salunke (2013) and Biswas (2016). Peaks of individual protein fractions on CE electrophoretogram for each sour cream including low molecular weight peptides (LMW), other peptides (OP), α -lactalbumin (α -LAC), β -lactoglobulin (β -LG), β -casein (β -CN), α S₁ casein (α S₁-CN), α S₂ casein (α S₂-CN), and κ -casein (κ -CN) were identified, and the area was calculated as percentage of total area of identified positive peaks according to Salunke (2013) and

Biswas (2016). Proteolysis of sour cream was indicated by calculating LMW, and OP identified on CE electrophoretogram of that respective sour cream sample. LMW and OP are produced from the hydrolysis of serum protein and casein present in sour cream. Higher the values of LMW and OP are indicating more proteolysis induced in sour cream. The example electrophoretogram of sour cream with identified peaks of individual protein fractions is shown in Fig. 1.

Cryo-Scanning Electron Microscopy

Cryo-scanning electron microscopy (Cryo-SEM) was used to study the microstructure of sour creams using a Zeiss Supra 40 high-resolution FE-SEM (Zeiss, Carl Zeiss Ltd., Hertfordshire, UK). An intact piece of sour cream was placed over an edge mounting, followed by rapid freezing using liquid nitrogen in a sealed vacuum chamber. The frozen sour cream specimen was gently transferred via airlock specimen holder to a preparation chamber (set at -150°C) and fractured to expose the fresh surface, followed by sublimation at -95°C for 5 min to remove the accumulated frost on a specimen. After sublimation, a specimen was subjected to a platinum sputter coating for 120 s. The coated specimen of sour cream was moved carefully in a cryo-SEM chamber and acclimated to a microscope environment for 5 min prior to microscopic observation. The microstructure of sour cream was evaluated at 2.5 K magnification with an aperture size of 30 µm on the 30th day of storage.

Graininess

The graininess of sour cream was measured according to Meletharayil et al., (2016). Approximately 1 g of sour cream was dispersed in 5 mL of water. A dispersion of

sour cream was gently mixed for 5 min without disturbing its integrity. Microscopic evaluation and image analysis were performed as per the method described by Meletharayil et al., (2016). Grains with perimeter > 1 mm were enumerated and results were expressed as a number of grains per g of sour cream. The graininess of sour cream was measured on the 30th day of storage.

Rheological Measurements

A stress-controlled rheometer (ATS Rheosystems, Rheologica Instrument Inc., Bordentown, NJ, USA) was used to perform the rheological measurements. A plate and plate assembly (diameter 30 mm) with a gap of 2 mm were used for flow behavior analysis and dynamic oscillatory measurement. Fine sandpaper (400 grit, Wetordry, 3M) was cut and glued to the upper plate of a rheometer to prevent a sample slippage. Sour cream samples were gently stirred for 30 times using a spoon for the homogeneity of a sample just prior to rheological analysis. Rheological measurements were performed at 4°C for 1, 15, and 30 days stored sour cream.

Flow curves were obtained by subjecting a sour cream sample of 1.6 g for pre-shear at 10 s^{-1} for 20 s, followed by the equilibrium period of 20 s. The shear rate was increased from 1 to 250 s^{-1} in 25 linear steps with a delay time of 10 s and integration time of 1 s then decreased from 250 to 1 s^{-1} in 25 liner steps with a delay time of 10 s and integration time of 1 s. The upward curve was fitted to the power law model to calculate flow behavior index (n) and consistency coefficient (κ). The apparent viscosity (η_a) was compared at 250 s^{-1} from the upward curve for all sour cream samples. Area of hysteresis

loop (AOH; the area between the upward and downward shear rate vs. shear rate curves) was calculated using a Rheo Explorer software-5.0.

The frequency dependency on the elastic and viscous modulus of sour cream was determined by first conducting a stress sweep measurement at a constant frequency of 0.15 Hz. A frequency sweep was performed at a constant stress of 1 Pa, with frequency ranging from 0 to 7 Hz in 50 steps to evaluate the viscoelastic properties of sour cream as per method described by Purohit et al. 2009. Values of viscoelastic moduli were recorded as a function of frequency and compared at 1 Hz for all sour cream samples. The slope of log-log plots of viscoelastic moduli was calculated to determine frequency dependency of the moduli.

Textural Analysis

Hardness and firmness of sour cream were measured by a large deformation penetration test using TA-XTplus Texture Analyzer (Texture Technologies Corp., South Hamilton, MA, USA) as per method described by Meletharayil et al. 2016. A penetration test was performed on approximately 100 g of sour cream in sterile plastic cups using a 0.5-inch diameter cylindrical probe with a test speed and post-test speed of 0.5 mm. s⁻¹ to the distance of 15 mm using a 5-Kg load cell. The maximum attained positive force during a test was expressed as hardness, and the total positive area was expressed as firmness of sour cream. The textural analysis was performed at 4°C on 1, 15, and 30 days stored sour cream.

Water-Holding Capacity

Water-holding capacity (WHC) of sour cream was performed according to a protocol described by Hassan et al. (1996). A cultured sour cream mix of 20 g from each treatment was transferred in 50 ml centrifuge tubes and fermented, followed by cooling and storage at 4°C. Water-holding capacity [WHC (%)] of sour cream was expressed as the ratio of the weight of pellet obtained after centrifugation to the initial weight of the sour cream. WHC (%) of sour cream was measured at 4°C on the 1st, 15th, and 30th day during storage.

Phospholipids and Volatile Free Fatty Acids Content

Phospholipids content of sour cream was analyzed as per the method described by Le et al., (2011) using UPLC (ACQUITY UPLC H-Class system, Waters Corporation, Milford, MA) with ELS detector. A HILIC column (Particle size: 1.7 µm, 2.1 x 150 mm) was used for phospholipids separation. Phospholipids content was evaluated for sour cream added with a phospholipid degrading *Bacillus licheniformis* DBC1 (T2 and T4) and a control sour cream (T1) on the 30th day of storage.

Volatile free fatty acids analysis was performed using HS-SPME GC-MS on Agilent 6890/5973 GC-MS (Agilent Technologies, Santa Clara, CA) with a ZB-Wax [30 m (L) x 0.25 mm (ID) x 0.25µm (FT)] column (Lund and Hølmer, 2001). Volatile free fatty acids content was measured for sour cream added with *B. licheniformis* DBC1 (T3 and T4) which was identified as a lipolytic *Bacillus* spp. using a spirit blue agar method and for sour cream without added any *Bacillus* which served as a control (T1). Volatile free fatty acids content of sour cream was performed on the 30th day of storage.

Sensory Analysis

Descriptive flavor and texture profiles were performed on sour cream (Shepard et al., 2013) with a 0 to 15-point universal intensity scale using the SpectrumTM method (Meilgaard et al., 1999; Drake and Civille, 2003). Sensory panel (n=6, 6 females, ages 22-46 years) with more than 200 h experience of descriptive analysis of dairy flavors and textures had evaluated sour cream samples. Sensory evaluation was performed on 30 days stored sour cream samples.

Statistical Analysis

Results were analyzed by ANOVA using R-3.4.0 software (R Core Team, Vienna, Austria) and significant results were indicated by $P < 0.05$. LSD test was used to determine the significant difference between the means. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Microbial Counts

Sour cream blend added with *Bacillus* (T2, T3, and T4) along with a control (T1; without added any *Bacillus*) were fermented at 26°C to a pH of 4.6 ± 0.05 and starter cultures count, *Bacillus* count, and pH were monitored during fermentation (Fig. 2). The growth pattern of starter culture during fermentation was found to be similar in sour cream with all treatments but the starter culture count was found to be higher (Table 2) at the end of fermentation in sour cream added with *Bacillus* (T2, T3, and T4) when compared with a control sour cream (T1). Moreover, sour cream blend fermented with

Bacillus (T2, T3, and T4) had reduced the fermentation time in than a control (T1). In a particular study, yogurt was made from milk treated with proteolytic enzymes which resulted in yogurt with a lower fermentation time (Gassem and Frank, 1991). In addition to the lower fermentation time, researchers have indicated that proteolytic activity of milk by proteases presumably increased readily available nitrogen and one might expect to find greater numbers of starter culture in a protease treated product. *Bacillus* spp. that we have used in our study were found to be proteolytic in nature. Their proteolytic activity could have increased readily available nitrogen for sour cream starter culture during fermentation and consequently, resulted in reduced fermentation time and a higher starter culture count in *Bacillus* treated sour cream at the end of fermentation.

Bacillus count was increased about a log cfu/g from the initial *Bacillus* count during fermentation in *Bacillus* added sour cream (T2, T3, and T4), followed by reduction in a count was observed with a decrease in pH in the range of 5.8 to 5.0, and found to be absent in 1st dilution of sour cream during microbial analysis at the end of fermentation (Fig. 2). Yazdany and Lashkari (1975) studied the effect of pH on the sporulating strains of *B. stearothermophilus* in a sporulating medium and found that below pH 5.0, the growth of the strains of *B. stearothermophilus* was completely suppressed. This is due to the enzymes of *B. stearothermophilus* which are responsible for utilizing organic acids during spore production from vegetative cells may not be induced or may be inactive at low pH values, and therefore, sporulation does not occur and consequently, completely suppressed their growth at low pH. Similar conclusion can be predicted for the spore-forming *Bacillus* spp. used in our study. Moreover, *Bacillus* spp. that we have used in this study are different than *B. stearothermophilus* and if we

presume that selected *Bacillus* spp. were sporulated at low pH then the number of spores which were produced during fermentation could be low in number and hence, found to be absent in 1st dilution of sour cream during microbial analysis at the end of fermentation.

The growth of *Bacillus* was observed to be higher during fermentation in sour cream blend inoculated with individual *Bacillus* spp. when compared to sour cream blend added with a cocktail of *B. subtilis* DBC and *B. licheniformis* DBC1, indicating the competitive growth of *B. subtilis* DBC and *B. licheniformis* DBC1 during fermentation in a treatment T4 and consequently, resulted in a lower number of *Bacillus* count during fermentation. In between individual *Bacillus* added sour cream blend (T2 and T3), sour cream blend added with *B. subtilis* DBC was found with a higher *Bacillus* count during fermentation as compared to sour cream blend added with *B. licheniformis* DBC1. This is due to the initial *Bacillus* count of sour cream blend added with *B. subtilis* DBC was higher. Moreover, *B. subtilis* DBC was observed with high proteolytic activity (NCN content: 0.182%) at sour cream fermentation temperature. The higher proteolytic activity of *B. subtilis* DBC could have increased readily available nitrogen in a sufficient amount during fermentation which consequently, enhanced their growth during fermentation. Storage study revealed that starter culture count was decreased approximately from log 8.0 to 8.5 cfu/g to log 4.0 to 4.5 cfu/g with reduction in pH in all sour cream samples stored at 4°C (Fig. 3), whereas *Bacillus* count was observed to be absent in 1st dilution in *Bacillus* added sour cream during storage. The viability of starter culture could be affected due to low pH and consequently, resulted in a reduction of starter culture count during storage.

Measurement of Proteolysis

Non-protein nitrogen [NPN (%)] content of sour cream with different treatments on 1st, 15th, and 30th day of storage is shown in Fig. 4. The proteolytic activity of *Bacillus subtilis* DBC in sour cream blend during fermentation resulted in a higher ($P < 0.05$) NPN (%) content as compared to sour cream without added any *Bacillus* (T1). *Bacillus* added sour cream, T2 and T4 were also observed with an increased NPN (%) content as compared to a control (T1), but results were found to be non-significant ($P > 0.05$). This can be expected because, in our preliminary study, *B. subtilis* DBC had observed with high proteolytic activity (NPN content: 0.182%) as compared to *B. licheniformis* DBC1 (NPN content: 0.092%) at sour cream fermentation temperature. Therefore, the proteolysis induced by *B. subtilis* DBC during sour cream fermentation could have increased NPN content and hence, resulted with a higher NPN (%) in 1st day stored sour cream. Moreover, the value of NPN (%) of 1st day stored sour cream, indicating the limited level of proteolysis induced by *Bacillus* spp. during fermentation (T2, T3, and T4). Storage study of sour cream revealed that NPN (%) content of sour cream with all treatments was increased throughout the storage, but the significant ($P < 0.05$) difference in NPN (%) was observed in sour cream stored for 15 days with respect to one day stored sour cream, thereafter, there was no significant ($P > 0.05$) difference was monitored between 15 and 30 days stored sour cream for all treatments. This is due to a reduction in starter culture count during storage (Fig. 3) which might also affect their proteolytic activity during storage. The proteolytic activity of starter culture could be sufficient to increase the NPN content of sour cream in first 15 days of storage, whereas after 15 days of storage, the viability of starter culture might not be sufficient to increase the NPN

content of sour cream and consequently, resulted with a non-significant difference in NPN (%) between 15 and 30 days stored sour cream.

The individual protein fractions present in sour cream quantified by performing capillary gel electrophoresis (CE) are shown in Table 3. A total of the percentage area of low molecular weight peptides (LMW) and other peptides (OP) was indicated as proteolysis was found to be higher in *Bacillus* added sour cream with respect to sour cream without added any *Bacillus*. Results of individual protein fractions (%) of 1st day stored sour cream added with *Bacillus* (T2, T3, and T4) showed lower values of κ -CN which indicates that sour cream blend added with *Bacillus* had hydrolyzed primarily κ -CN during fermentation, whereas there was no impact observed on β -CN during fermentation. Results of protein profile of sour cream during storage indicates that proteolysis was continued in all sour cream samples even at refrigerated storage. This finding can be correlated to the explanation had been made for increased NPN (%) content of sour cream during storage.

Microstructure and Graininess

The microstructure of sour cream was evaluated using cryo-scanning electron microscopy (Cryo-SEM) for each treatment is shown in Fig. 5. The images of microstructure clearly indicate that *Bacillus* added sour cream had resulted in the aggregated protein network with extensive cross-linking in sour cream with treatments, T3 and T4. *Bacillus* spp. had hydrolyzed κ -CN during fermentation which could be resulted in steric destabilization of casein micelles and consequently, produced an aggregated protein network in sour cream. Moreover, the limited level of proteolysis

induced by *Bacillus* spp. might have resulted in inter- and intra- molecular interactions of protein which may be also responsible for aggregated protein network in *Bacillus* added sour cream.

The graininess of sour cream was defined as the number of grains with a perimeter > 1 mm per g of sour cream (Table 4). Sour cream treated with *Bacillus* spp. (T2, T3, and T4) had observed with a higher number ($P < 0.05$) of grains per g of sour cream as compared to a control (T1) with the highest value of 272 grains/g of sour cream was observed for *B. subtilis* DBC treated sour cream. The representative stereo microscopic images also revealed that *Bacillus* added sour cream had a higher grainy structure and the level of graininess was found to be higher for sour cream with treatments, T3 and T4. These findings could be correlated with results which were observed during the microstructure evaluation of sour cream using cryo-SEM. The aggregated protein network induced by *Bacillus* could have resulted in the development of grains and consequently, more grainy structure was observed in sour cream added with *Bacillus* spp. during stereo microscopic evaluation. Moreover, graininess can be contributed by the fusion of small crystals of fat during refrigerated storage. Heertje et al. (1988) observed that many small crystals of fat are formed and undergo to crystal-crystal interactions upon cooling.

Rheological Properties

Rheological properties of sour cream added with *Bacillus* (T2, T3, and T4) and a control sour cream (T1) are shown in Table 5. All sour cream samples were characterized for flow characteristics by fitting the flow data into a power law model and calculated for

an area of hysteresis loop (AOH) using a Rheo Explorer software-5.0. The AOH was observed significantly ($P < 0.05$) higher for all sour cream samples added with *Bacillus* (T2, T3, and T4) as compared to a control sour cream (T1), whereas flow behavior index (n) of sour cream was found to be significantly ($P < 0.05$) lower for treatments T2, T3, and T4, as compared to T1. The AOH measures the extent of structure breakdown during shearing (Hassan et al., 1996). Results of AOH for *Bacillus* added sour cream (T2, T3, and T4) indicating more structure breakdown during shearing action as compared to a control sour cream (T1) and *B. subtilis* DBC added sour cream (T3) was observed with the highest structure breakdown (AOH: 7201 Pa. s⁻¹) among the *Bacillus* treated sour creams (T2, T3, and T4). As we discussed earlier, selected *Bacillus* spp. are proteolytic, and *B. subtilis* DBC had shown a higher proteolytic activity (NCN content: 0.182%) as compared to *B. licheniformis* DBC1 (NCN content: 0.092%) at sour cream fermentation temperature. The type and interaction induced by *Bacillus* in the protein network in *Bacillus* added sour cream might be not recoverable upon shearing and consequently, observed with high AOH values. Moreover, the nature and type of interactions in the protein network induced by *B. subtilis* DBC could be different than *B. licheniformis* DBC 1, resulting in the highest value of AOH. The lower n values for sour cream added with *Bacillus* spp. (T2, T3, and T4) than sour cream without added *Bacillus* (T1) indicate more deviation of flow of *Bacillus* added sour cream from Newtonian behavior (Purohit et al., 2009). *Bacillus* added all sour cream had a greater effect on the flow behavior characteristics with a higher consistency coefficient (κ), and apparent viscosity (η_a) at 250 s⁻¹. The relatively high κ in *Bacillus* added sour cream (T2, T3, and T4), indicating firmer texture due to the extensive cross-linking induced by inter- and intra- molecular

interactions in the protein network. In a particular study, researchers had observed that limited level of proteolysis in UHT milk had increased the viscosity. This is due to hydrolysis of caseins, releasing the β -Lg- κ -CN complex, formed during heat treatment, from the micelles. The released complex subsequently produced the aggregates and forms a three-dimensional network of cross-linked proteins, which increase the viscosity (McMahon, 1996). A similar explanation can be applied for sour cream added with *Bacillus*. Limited level of proteolysis induced by *Bacillus* spp. could have released β -Lg- κ -CN complex which was formed during heat treatment of sour cream blend, from the micelles and subsequently, released complexes had aggregated and formed a three-dimensional network of cross-linked proteins, which could have taken place in serum phase and consequently, resulted in increased κ , and η_a values in *Bacillus* added sour cream. Among all sour cream treatments, *B. subtilis* added sour cream had a greater impact on rheological properties of sour cream including AOH, n , κ , and η_a .

Rheological properties of sour cream also influenced during storage at 4°C. The AOH, κ , and η_a values were increased significantly ($P < 0.05$) of sour cream added with *Bacillus* spp. and control upon storage whereas no significant ($P > 0.05$) changes was observed on n values. This is due to the proteolytic activity was found to be slower (less increase in NPN content) during storage. Heertje et al. (1988) observed that many small crystals of fat are formed upon cooling that induces the number of crystal-crystal interactions. This phenomenon can influence the rheological properties milk-fat based products (Heertje et al., 1988; Rønholt et al., 2012; Rønholt et al., 2014). Similarly, the fusion of fat crystals could have increased the rigidity of sour cream structure during

refrigerated storage and thus, observed with the different rheological properties of sour cream upon storage at 4°C.

Viscoelastic Properties

The values of elastic (G'), viscous (G''), and complex (G^*) moduli of sour cream added with *Bacillus* (T2, T3, and T4) and a control (T1) at 1 Hz frequency are shown in Table 6. The elastic and viscous moduli are proportional to the number of bonds formed in a gel network (Lucey et al., 2003). The elastic modulus is a measure of strong nonrelaxing bonds, whereas the viscous modulus is associated with weak relaxing bonds (Roefs et al., 1985; Lankes et al., 2003). A higher ($P < 0.05$) viscoelastic moduli (G' , G'' , and G^*) was observed for sour cream added with *Bacillus* (T2, T3, and T4) as compared to sour cream without added *Bacillus* (T1). This can be correlated with a dense protein network was observed in sour cream added with *Bacillus* spp. induced by various inter- and intra- molecular protein interactions due to a limited level of proteolysis. Moreover, *Bacillus* added sour cream could be compared to rennet acid induced milk gel where rennet hydrolyzes κ -CN instead of *Bacillus*, whereas sour cream without added any *Bacillus* can be compared to acid milk gel. Lucey et al., (2000) reported that milk gel formed by rennet acid coagulation had high viscoelastic properties when compared to only acid induced milk gel. Phase angle indicates the nature and type of interaction forces take place during structure formation (Purohit et al., 2009). There was no difference ($P > 0.05$) in phase angle values among sour cream added with *Bacillus* (T2, T3, and T4) and a control (T1). Because of the limited level of proteolysis occurred in sour cream added with *Bacillus*, phase angle could not be able to differentiate the nature and type of interaction forces occurred in sour cream added with *Bacillus* spp. (T2, T3, and T4) and

without any *Bacillus* (T1) at a significant level. A positive value of the slope of log viscoelastic moduli vs. log frequency (Hz) for all sour cream manufactured with different treatments, indicating frequency dependence structure of sour cream. The slope of the elastic modulus (G') vs. Hz frequency curve was found to be higher ($P < 0.05$) for sour cream added with *Bacillus subtilis* DBC (T3), and a cocktail of both *Bacillus* spp., *B. subtilis* DBC and *B. licheniformis* DBC1 (T4). The extensive cross-linking in protein network by *Bacillus* attributed the high-frequency dependence of sour cream structure.

A value of viscoelastic moduli (G' , G'' , and G^*), and slope of log viscoelastic moduli (G' , G'' , and G^*), vs. log frequency (Hz) of sour cream with all treatments (T1, T2, T3, and T4) except phase angle, were increased significantly ($P < 0.05$) during storage at 4°C. A similar increased G' value was observed in cream during refrigerated storage (Rønholt et al., 2012; Rønholt et al., 2014). This can be correlated with the explanation had been made for rheological properties of a sour cream.

Textural Properties

Textural properties such as hardness and firmness values of sour cream are shown in Table 7. Sour cream added with *B. subtilis* DBC (T3), and a cocktail of *Bacillus* spp., *B. subtilis* DBC and *B. licheniformis* DBC1 (T4) had higher ($P < 0.05$) hardness and firmness values, whereas there was no significant ($P > 0.05$) difference in textural properties of sour cream added with *B. licheniformis* DBC1 (T2), and a control sour cream without added any *Bacillus* (T1). There was significant ($P < 0.05$) impact of storage on textural properties of a sour cream with all treatments (T1, T2, T3, and T4). Hardness and firmness values of sour cream had increased over a period of storage.

Values of hardness and firmness of sour cream were increased significantly ($P < 0.05$) from 1st day to 15th day of storage, consequently there was no significant ($P > 0.05$) change in textural properties between 15 and 30 days stored sour cream. These results can be correlated with the results obtained from the measurement of rheological and viscoelastic properties, and microstructure evaluation of sour cream.

Water-Holding Capacity

As shown in Fig. 6, a higher ($P < 0.05$) water-holding capacity [WHC (%)] was observed for sour cream added with *B. subtilis* DBC (T3), whereas WHC (%) of sour cream added with *B. licheniformis* DBC1 (T2), and a cocktail of both *Bacillus* spp. (T4) had no significant ($P > 0.05$) difference when they compared to a control (T1). This is due to the extensive cross-linking of protein network in sour cream induced by *B. subtilis* had produced very small pores in sour cream structure which led to increasing the WHC (%) of sour cream. WHC (%) of sour cream with all treatments was increased during storage, but results were non-significant ($P > 0.05$).

Phospholipids and Volatile Free Fatty Acids Content

Phospholipids content of sour cream (Table 8) was indicated as total phospholipids (%) content and individual phospholipid (%) including phosphatidyl inositol, phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl serine and sphingomyelin of total phospholipids (%). Results indicate that there was no significant ($P > 0.05$) difference observed in phospholipids content of sour cream added with phospholipid degrading *B. licheniformis* DBC1 (T2 and T4) and a control sour cream (T1). Volatile free fatty acids including short chain fatty acids [butyric acid (C4:0),

caproic acid (C6:0), and caprylic acid (C8:0)] and long chain fatty acids [palmitic (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3)] were evaluated for sour cream (Table 9) added with a lipolytic *B. licheniformis* DBC1 (T2 and T4) and sour cream without added any *Bacillus* (T1). Lipolytic activity of *B. licheniformis* DBC1 had not resulted in any significant ($P > 0.05$) impact on volatile free fatty acids profile of sour cream (T2 and T4) with respect to control (T1). This can be due to lower inoculation level (approximately 10^3 cfu/mL) of phospholipase and lipase producing *B. licheniformis* DBC1 in the sour cream blend. Phospholipase and lipase produced from *B. licheniformis* at that inoculation level could not be sufficient to degrade phospholipids and fat at the detected level and hence, observed with non-significant results of phospholipids and volatile free fatty acids of sour cream added with *B. licheniformis* DBC1 (T2 and T4) and a control (T1). Most research indicates that fairly high total bacteria count ($>10^6$ cfu/mL) in raw milk are needed to cause defects in most processed dairy products (Murphy et al., 2016). Moreover, the growth of *B. licheniformis* DBC1 was suppressed at lower pH during fermentation (Fig. 2) which indicates that there might be insufficient time for *B. licheniformis* DBC1 to grow enough during fermentation and degrade phospholipids and fat present in the sour cream.

Sensory Evaluation

Sour cream was evaluated for descriptive flavor profiles (Fig. 7A) by trained sensory panelists including cooked/milky, dairy sour, milkfat, cabbage/cardboard, sweet taste, sour taste, astringency, and overall aroma intensity using the universal intensity scale from 0 to 15 by spectrum method (Mailgaard et al., 1999). According to (Drake, 2004), most of the sour cream flavors fall between 0 to 5 on a 0-15 universal intensity

scale. In this study, the scores for selected flavors had also found between 0 to 5 on a scale for sour cream with all treatments. There were no differences observed for sour cream added with *Bacillus* and a control. Moreover, sensory panelists did not taste any bitterness in *Bacillus* treated sour cream. This is due to the proteolysis induced by *Bacillus* spp. was not at higher level. Moreover, the hydrolysis of β -CN is primarily responsible for bitterness in dairy products (Fernandez-Garcia, 1994). In our study, we did not observe the degradation of β -CN in sour cream by selected *Bacillus* spp.

There were differences observed in descriptive texture profiles of sour cream (Fig. 7B) manufactured with *Bacillus* and a control sour cream. A higher firmness was observed in all *Bacillus* added sour cream and the highest value of firmness was observed for sour cream added with *B. subtilis* DBC. This finding also supports to the results obtained from the measurement of texture properties of sour cream which were analyzed for hardness and firmness by a penetration test (Table 7). Moreover, sensory panelists had graded sour cream added with *B. subtilis* DBC with the highest value of graininess. Similar results also observed for *B. subtilis* DBC added sour cream when evaluated for grains per g of sour cream by a microscopic evaluation (Table 4).

CONCLUSION

The contamination of common spore-forming bacilli at lower inoculation level (10^3 cfu/g) in sour cream blend during fermentation had brought rheological, texture, physicochemical, and microstructure changes in sour cream even though *Bacillus* spp. had grown and survived for few hours during fermentation until the pH was low enough to inhibit their growth. Variation in properties of sour cream added with *Bacillus* spp. was

primarily due to a limited level of proteolysis induced by *Bacillus* spp. Moreover, changes in properties of *Bacillus* added sour cream may be brought by mainly *B. subtilis* DBC due to its high proteolytic activity, therefore, sour cream added with *B. subtilis* DBC (T3) and a cocktail of *Bacillus subtilis* DBC and *B. licheniformis* DBC1 (T4) had observed with the highest influenced properties as compared to sour cream added with *B. licheniformis* DBC1 (T2). There was no synergistic effect of *B. subtilis* DBC, and *B. licheniformis* DBC1 (T4) observed on sour cream properties. Spiking of sour cream with *Bacillus* spp. at 10^3 cfu/ml during fermentation did not induce any lipolysis or flavor defects such as bitterness in sour cream after 30 days of storage. Limited level of proteolysis by *Bacillus* spp. resulted in a dense microstructure of sour cream with increased graininess. Storage study revealed that lower proteolytic activity and fusion of fat crystals during refrigerated storage had brought textural changes in sour cream. The present work indicates that contamination in cultured dairy products like sour cream with proteolytic *Bacillus* spp. may result in inferior texture and cause the batch to batch variations during manufacturing. To our knowledge, this is the first report to show the impact of *Bacillus* on sour cream texture. Strategies to control spore-forming bacilli in cultured dairy products need to be developed.

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Table 1. Design of experiment consisting a total of four sour cream treatments

Treatments	Sour cream treatments			
	T1	T2	T3	T4
Description	Sour cream blend inoculated with sour cream starter cultures (0.01%, w/w) but without added any <i>Bacillus</i>	Sour cream blend inoculated with sour cream starter cultures (0.01%, w/w) and added with <i>B. licheniformis</i> DBC1 (approximately 10^3 cfu/g)	Sour cream blend inoculated with sour cream starter cultures (0.01%, w/w) and added with <i>B. subtilis</i> DBC (approximately 10^3 cfu/g)	Sour cream blend inoculated with sour cream starter cultures (0.01%, w/w) and a cocktail of <i>B. licheniformis</i> DBC1 and <i>B. subtilis</i> DBC (approximately a total of 10^3 cfu/g)

Table 2. pH, starter culture count, and fermentation time of sour cream

Treatments	pH ¹	Starter culture count (Log cfu/g) ¹	Fermentation time (min) ¹
T1	4.61	8.43	812
T2	4.62	8.49	799
T3	4.60	8.72	778
T4	4.61	8.60	787

¹ n=3

T1 = sour cream blend without added any *Bacillus*; T2 = sour cream blend added with *B. licheniformis* DBC1 (10^3 cfu/g); T3 = sour cream added with *B. subtilis* DBC (10^3 cfu/g); T4 = sour cream added with *B. licheniformis* DBC1 and *B. subtilis* DBC (a total of 10^3 cfu/g)





Table 3. Protein fractions (%) present in sour cream observed on capillary gel electrophoresis electrophoretogram

Time interval (days)	Treatments	Protein fractions (%) ¹						
		Proteolysis (LMW + OP)	α -LAC	β -LG	β -CN	α S ₁ -CN	α S ₂ -CN	κ -CN
1 st	T1	4.09	3.59	9.06	29.64	38.42	10.93	3.64
	T2	4.53	3.63	8.61	31.25	37.95	10.31	3.22
	T3	4.69	3.82	8.70	31.27	37.81	9.43	3.06
	T4	4.13	3.34	8.51	29.92	38.31	11.33	3.11
15 th	T1	4.01	3.26	9.51	33.62	35.24	10.80	3.40
	T2	4.43	3.20	8.20	32.48	37.88	10.28	3.10
	T3	5.42	3.01	7.87	32.00	38.56	10.93	2.21
	T4	4.82	3.20	8.94	32.81	37.19	10.29	2.76
30 th	T1	4.71	3.27	9.47	33.11	34.70	10.48	3.37
	T2	5.60	3.12	7.68	31.98	37.34	10.19	3.05
	T3	6.41	2.94	7.91	31.35	37.87	10.53	2.14
	T4	5.57	3.72	8.54	32.31	36.99	10.03	2.80

¹ n=3,

T1 = sour cream blend without added any *Bacillus*; T2 = sour cream blend added with *B. licheniformis* DBC1 (10³ cfu/g); T3 = sour cream added with *B. subtilis* DBC (10³ cfu/g); T4 = sour cream added with *B. licheniformis* DBC1 and *B. subtilis* DBC (a total of 10³ cfu/g)

Table 4. Representative stereo microscopic images and graininess of sour cream

Parameters	Treatments			
	T1	T2	T3	T4
Representative stereo microscopic images of sour cream ²				
Grains/g of sour cream ^{1,2}	23 ^c	171 ^b	272 ^a	185 ^b

^{a-c} Means in the same row without common superscripts are significantly different ($P < 0.05$)

¹ n=3

² Analysis was performed on 30th day of storage

T1 = sour cream blend without added any *Bacillus*; T2 = sour cream blend added with *B. licheniformis* DBC1 (10^3 cfu/g); T3 = sour cream added with *B. subtilis* DBC (10^3 cfu/g); T4 = sour cream added with *B. licheniformis* DBC1 and *B. subtilis* DBC (a total of 10^3 cfu/g)

Table 5. Rheological properties of sour cream

Parameters	Time intervals (days)	Treatments			
		T1	T2	T3	T4
Area of hysteresis loop (Pa·s ⁻¹) ¹	1 st	4295 ^{Bb}	6074 ^{Ba}	7201 ^{Ba}	7193 ^{Ba}
	15 th	6567 ^{Ab}	7342 ^{Ab}	9775 ^{Aa}	7810 ^{Bb}
	30 th	7652 ^{Ac}	8276 ^{Abc}	10279 ^{Aa}	9149 ^{Aab}
Flow behavior index ^{1,3}	1 st	0.16 ^{Aa}	0.14 ^{Abc}	0.15 ^{Aab}	0.13 ^{Ac}
	15 th	0.18 ^{Aa}	0.15 ^{ABab}	0.14 ^{Ab}	0.14 ^{Ab}
	30 th	0.18 ^{Aa}	0.16 ^{Aab}	0.13 ^{Abc}	0.12 ^{Ac}
Consistency coefficient (Pa·s ⁿ) ^{1,3}	1 st	33.76 ^{Cc}	47.94 ^{Bb}	59.07 ^{Ba}	55.29 ^{Bab}
	15 th	47.77 ^{Bb}	60.60 ^{Aa}	68.38 ^{ABa}	62.02 ^{Ba}
	30 th	59.74 ^{Ac}	65.05 ^{Abc}	80.82 ^{Aa}	70.10 ^{Ab}
Apparent viscosity (Pa·s) ^{1,2}	1 st	0.29 ^{Bc}	0.34 ^{Bb}	0.41 ^{Aa}	0.39 ^{Ba}
	15 th	0.41 ^{Aa}	0.43 ^{Aa}	0.46 ^{Aa}	0.43 ^{Aba}
	30 th	0.42 ^{Aa}	0.47 ^{Aa}	0.48 ^{Aa}	0.49 ^{Aa}

^{A-C} Means of treatments in the same column between the days or ^{a-c} Means of treatments in the same row on the same day without common superscripts are significantly different ($P < 0.05$)

¹ n=6

² Values are at shear rate of 250 s⁻¹

³ Determined by fitting to the power law model

T1 = sour cream blend without added any *Bacillus*; T2 = sour cream blend added with *B. licheniformis* DBC1 (10³ cfu/g); T3 = sour cream added with *B. subtilis* DBC (10³ cfu/g); T4 = sour cream added with *B. licheniformis* DBC1 and *B. subtilis* DBC (a total of 10³ cfu/g)

Table 6. Viscoelastic properties of sour cream during storage

Parameters	Time interval (days)	Treatments			
		T1	T2	T3	T4
Elastic modulus ¹ (G', Pa)	1 st	466.4 ^{Bb}	781.6 ^{Ba}	843.8 ^{Ba}	886.2 ^{Ba}
	15 th	524.8 ^{Bb}	1068.1 ^{Aa}	1164.6 ^{Ba}	1432.6 ^{Aba}
	30 th	706.5 ^{Ab}	1140.8 ^{Aab}	1747.8 ^{Aa}	1697.7 ^{Aa}
Viscous modulus ¹ (G'', Pa)	1 st	113.2 ^{Bb}	188.8 ^{Ba}	203.6 ^{Ba}	210.2 ^{Ba}
	15 th	134.7 ^{ABb}	238.7 ^{Aab}	275.2 ^{Ba}	316.3 ^{Aa}
	30 th	153.2 ^{Ab}	267.5 ^{Aab}	434.3 ^{Aa}	412.2 ^{Aa}
Complex modulus ¹ (G*, Pa)	1 st	480.0 ^{Bb}	804.1 ^{Ba}	868.2 ^{Ba}	910.9 ^{Ba}
	15 th	541.7 ^{ABb}	1094.8 ^{Aa}	1197.0 ^{ABa}	1467.4 ^{Aa}
	30 th	722.9 ^{Ab}	1171.7 ^{Aab}	1801.8 ^{Aa}	1747.3 ^{Aa}
Phase angle ¹	1 st	13.6 ^{Aa}	13.6 ^{Aa}	13.5 ^{Aa}	13.4 ^{Aa}
	15 th	14.4 ^{Aa}	12.6 ^{Aa}	13.2 ^{Aa}	12.5 ^{Aa}
	30 th	12.2 ^{Aa}	13.2 ^{Aa}	14.0 ^{Aa}	13.6 ^{Aa}
Slope of log G' vs. log frequency (Hz) ¹	1 st	0.19 ^{Bb}	0.20 ^{Bb}	0.21 ^{Ba}	0.21 ^{Ba}
	15 th	0.23 ^{Ab}	0.23 ^{Ab}	0.25 ^{Aa}	0.23 ^{Ab}
	30 th	0.22 ^{ABb}	0.23 ^{Ab}	0.26 ^{Aa}	0.23 ^{Ab}
Slope of log G'' vs. log frequency (Hz) ¹	1 st	0.14 ^{Bb}	0.14 ^{Bb}	0.17 ^{Ba}	0.15 ^{Bab}
	15 th	0.15 ^{Bb}	0.14 ^{Bb}	0.19 ^{Aa}	0.15 ^{Bb}
	30 th	0.17 ^{Ab}	0.17 ^{Ab}	0.19 ^{Aa}	0.17 ^{Ab}
Slope of log G* vs. log frequency (Hz) ¹	1 st	0.21 ^{Ba}	0.22 ^{Ba}	0.22 ^{Ba}	0.22 ^{Ba}
	15 th	0.24 ^{Ab}	0.24 ^{Bb}	0.27 ^{Aa}	0.25 ^{Ab}
	30 th	0.25 ^{Ab}	0.25 ^{Ab}	0.26 ^{Ab}	0.26 ^{Ab}

^{A-B} Means of treatments in the same column between the days or ^{a-b} Means of treatments in the same row on the same day without common superscripts are significantly different ($P < 0.05$), ¹ n=6, ² Value measured at 1 Hz, T1 = sour cream blend without added any *Bacillus*; T2 = sour cream blend added with *B. licheniformis* DBC1 (10^3 cfu/g); T3 = sour cream added with *B. subtilis* DBC (10^3 cfu/g); T4 = sour cream added with *B. licheniformis* DBC1 and *B. subtilis* DBC (a total of 10^3 cfu/g)

Table 7. Textural properties of sour cream during storage

Parameters	Time interval (days)	Treatments			
		T1	T2	T3	T4
Hardness (Kg) ¹	1 st	0.116 ^{Bb}	0.125 ^{Bab}	0.137 ^{Ba}	0.131 ^{Ca}
	15 th	0.141 ^{ABc}	0.149 ^{ABbc}	0.171 ^{Aa}	0.161 ^{Bb}
	30 th	0.153 ^{Ab}	0.161 ^{Ab}	0.185 ^{Aa}	0.184 ^{Aa}
Firmness (Kg·s) ¹	1 st	1.338 ^{Bb}	1.414 ^{Bab}	1.616 ^{Ba}	1.538 ^{Ba}
	15 th	1.600 ^{ABb}	1.705 ^{ABab}	2.000 ^{Aa}	1.856 ^{Aa}
	30 th	1.756 ^{Ab}	1.858 ^{Aab}	2.148 ^{Aa}	2.122 ^{Aa}

^{A-C} Means of treatments in the same column between the days or ^{a-c} Means of treatments in the same row on the same day without common superscripts are significantly different ($P < 0.05$)

¹ n=6

T1 = sour cream blend without added any *Bacillus*; T2 = sour cream blend added with *B. licheniformis* DBC1 (10^3 cfu/g); T3 = sour cream added with *B. subtilis* DBC (10^3 cfu/g); T4 = sour cream added with *B. licheniformis* DBC1 and *B. subtilis* DBC (a total of 10^3 cfu/g)

Table 8. Phospholipids content^{1,2} of sour cream

Treatments	Total PL (%)	% of Total PL				
		PI	PE	PC	PS	SM
T1	2.69	ND	59.8	15.86	4.48	19.9
T2	2.59	ND	56.3	7.67	1.21	34.7
T4	2.80	ND	53.4	14.31	4.08	28.0

¹ n=2² Analysis was performed on 30th day of storage

T1 = sour cream blend without added any *Bacillus*; T2 = sour cream blend added with *B. licheniformis* DBC1 (10³ cfu/g); T4 = sour cream added with *B. licheniformis* DBC1 and *B. subtilis* DBC (a total of 10³ cfu/g)

PL-phospholipids, PI-phosphatidyl inositol, PE-phosphatidyl ethanolamine, PC-phosphatidyl choline, PS-phosphatidyl serine, SM-sphingomyelin, ND-not detected

Table 9. Volatile free fatty acids content^{1,2} of sour cream

Volatile free fatty acids (ppm)	Treatments		
	T1	T2	T4
<i>Short chain fatty acids</i>			
Butyric acid (C4:0)	1.82	0.90	1.49
Caproic acid (C6:0)	6.42	3.86	8.07
Caprylic acid (C8:0)	13.9	2.58	13.0
<i>Long chain fatty acids</i>			
Palmitic (C16:0)	11.0	2.82	8.59
Stearic acid (C18:0)	77.1	48.6	92.5
Oleic acid (C18:1)	0.56	0.20	0.50
Linoleic acid (C18:2)	1.58	0.26	0.62
Linolenic acid (C18:3)	0.37	0.27	0.66

¹ n=2² Analysis was performed on 30th day of storage

T1 = sour cream blend without added any *Bacillus*; T2 = sour cream blend added with *B. licheniformis* DBC1 (10^3 cfu/g); T4 = sour cream added with *B. licheniformis* DBC1 and *B. subtilis* DBC (a total of 10^3 cfu/g)

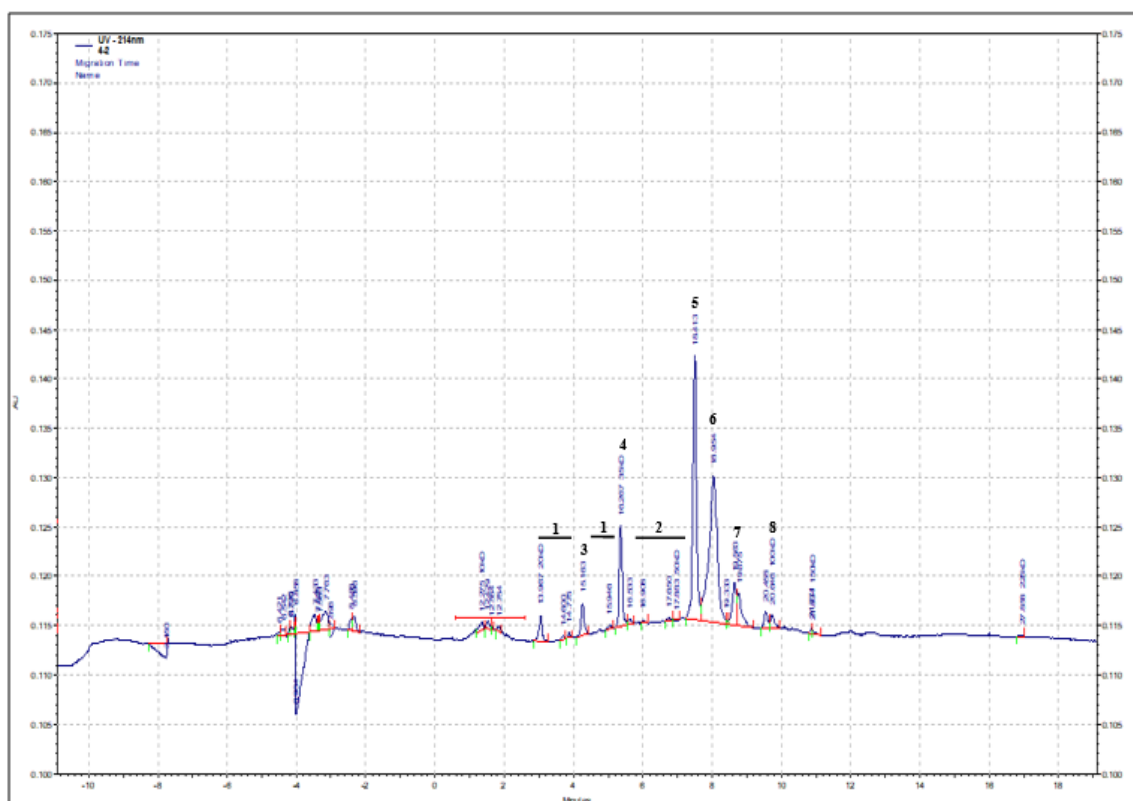


Figure 1. The example capillary gel electrophoresis (CE) electrophoretogram of sour cream. 1 = low molecular weight peptides (LMW); 2 = other peptides (OP); 3 = α -lactalbumin (α -LAC); 4 = β -lactoglobulin (β -LG); 5 = β -casein (β -CN); 6 = α S₁ casein (α S₁-CN); 7 = α S₂ casein (α S₁-CN); 8 = κ -casein (κ -CN)

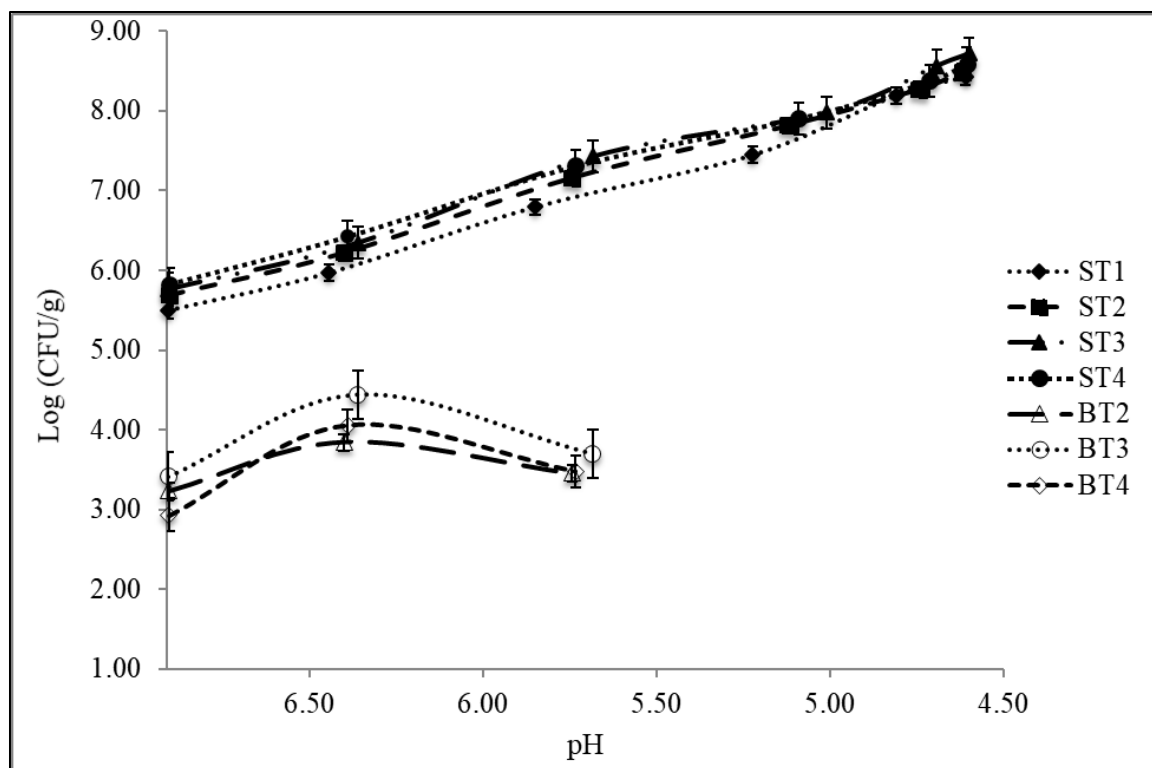


Figure 2. Starter culture count during fermentation of sour cream without added any *Bacillus* (ST1), added with *B. licheniformis* DBC1 at 10^3 cfu/g (ST2), added with *B. subtilis* DBC at 10^3 cfu/g (ST3) and a cocktail of *B. licheniformis* DBC1 and *B. subtilis* DBC at 10^3 cfu/g (ST4) and *Bacillus* count during fermentation of sour cream added with *B. licheniformis* DBC1 at 10^3 cfu/g (BT2), added with *B. subtilis* DBC at 10^3 cfu/g (BT3) and a cocktail of *B. licheniformis* DBC1 and *B. subtilis* DBC at 10^3 cfu/g (BT4). Error bars indicate SE and $n=3$.

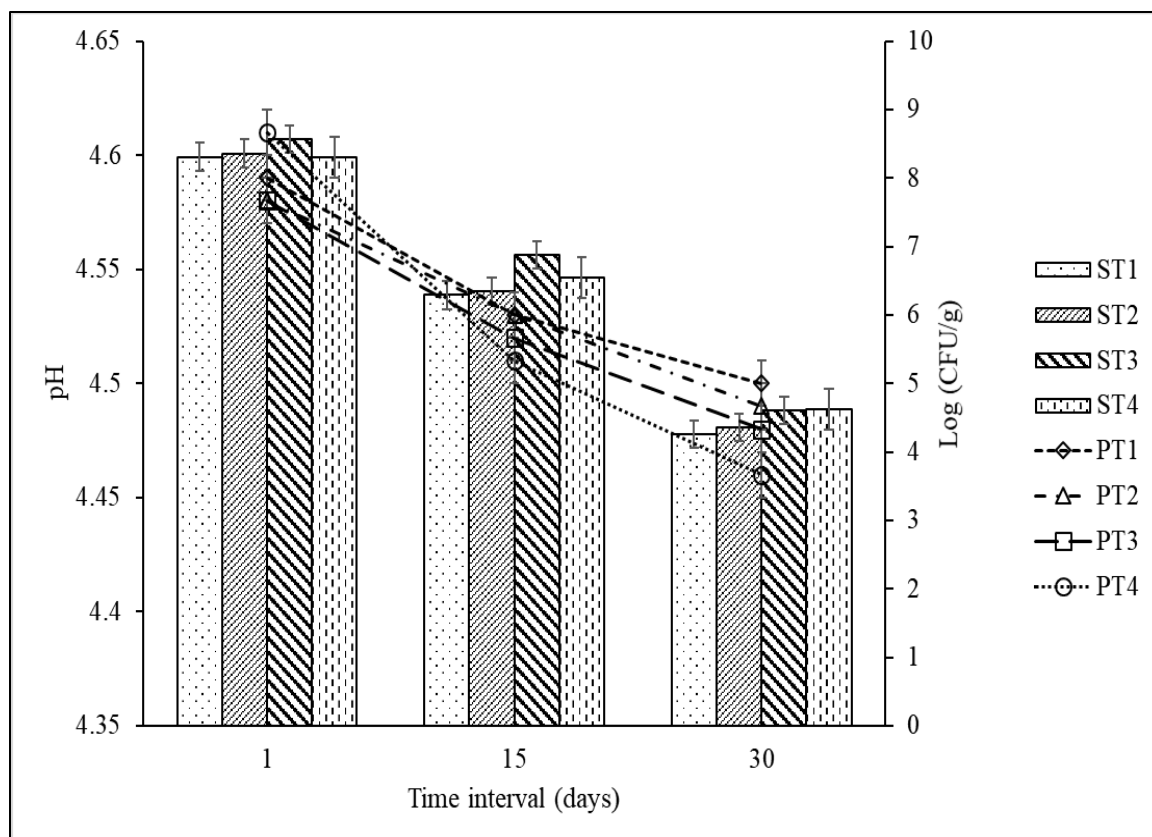


Figure 3. pH and starter culture count of sour cream during storage at 4°C. Lines indicate the pH of sour cream without added any *Bacillus* (PT1), added with *B. licheniformis* DBC1 at 10^3 cfu/g (PT2), added with *B. subtilis* DBC at 10^3 cfu/g (PT3) and a cocktail of *B. licheniformis* DBC1 and *B. subtilis* DBC at 10^3 cfu/g (PT4). Bars indicate the starter culture count of sour cream without added any *Bacillus* (ST1), added with *B. licheniformis* DBC1 at 10^3 cfu/g (ST2), added with *B. subtilis* DBC at 10^3 cfu/g (ST3) and a cocktail of *B. licheniformis* DBC1 and *B. subtilis* DBC at 10^3 cfu/g (ST4). Error bars indicate SE and n=3.

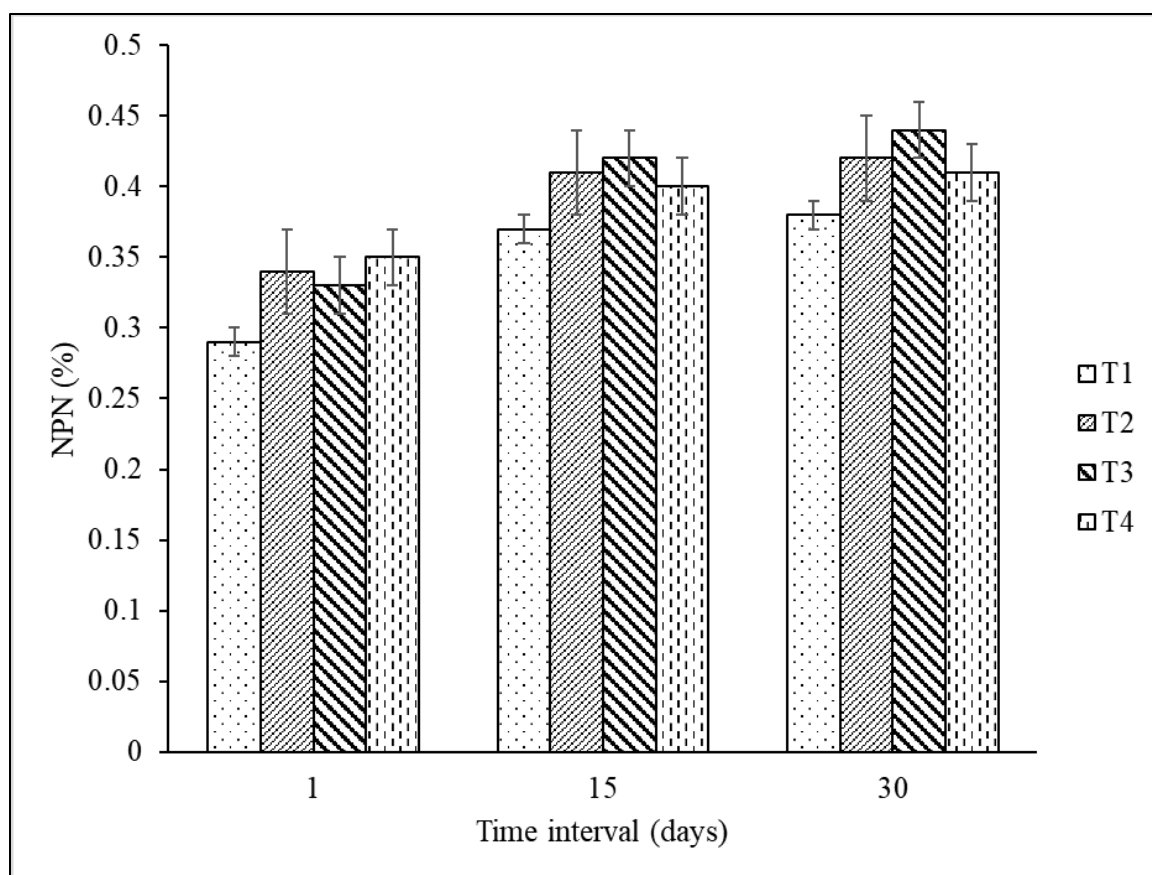


Figure 4. NPN (%; $N \times 6.38$) content of sour cream without added any *Bacillus* (T1), added with *B. licheniformis* DBC1 at 10^3 cfu/g (T2), added with *B. subtilis* DBC at 10^3 cfu/g (T3) and a cocktail of *B. licheniformis* DBC1 and *B. subtilis* DBC together at 10^3 cfu/g (T4) during storage at 4°C. NPN (%) content = NPN (%) \times 6.38. Error bars indicate SE and n=6.

Figure 5. Microstructure evaluation of sour cream using cryo-SEM without added any *Bacillus* (T1), added with *B. licheniformis* DBC1 at 10^3 cfu/g (T2), added with *B. subtilis* DBC at 10^3 cfu/g (T3) and a cocktail of *B. licheniformis* DBC1 and *B. subtilis* DBC at 10^3 cfu/g (T4) on 30th day of storage at 4°C. Microstructure of sour cream was evaluated at 2.5 K magnification with an aperture size of 30 μ m.

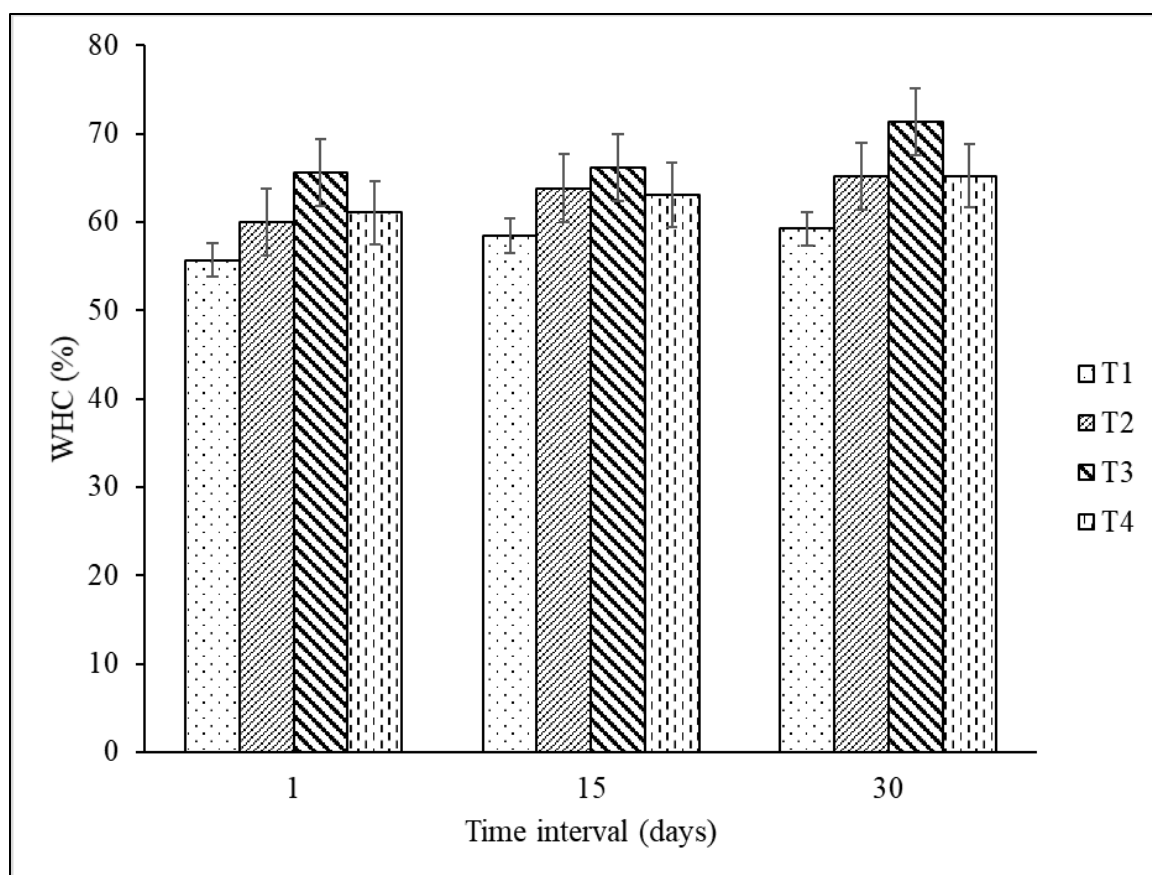


Figure 6. WHC (%) of sour cream without added any *Bacillus* (T1), added with *B. licheniformis* DBC1 at 10^3 cfu/g (T2), added with *B. subtilis* DBC at 10^3 cfu/g (T3) and a cocktail of *B. licheniformis* DBC1 and *B. subtilis* DBC at 10^3 cfu/g (T4) during storage at 4°C. Error bars indicate SE and n=6.

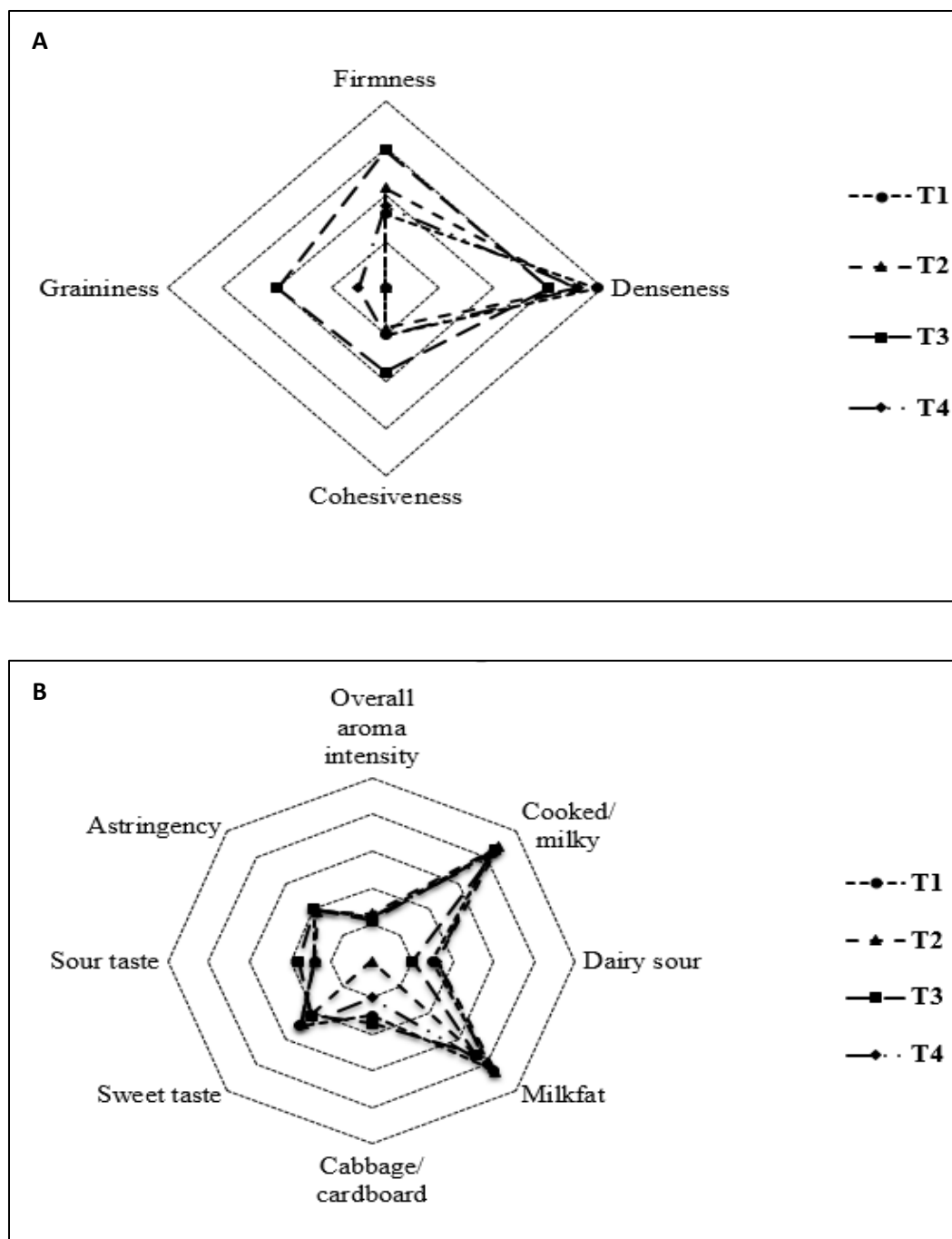


Figure 7. Descriptive flavor (A) and texture (B) profiles analysis of sour cream on a 0 to 15-point universal intensity scale without added any *Bacillus* (T1), added with *B. licheniformis* DBC1 at 10^3 cfu/g (T2), added with *B. subtilis* DBC at 10^3 cfu/g (T3) and a cocktail of *B. licheniformis* DBC1 and *B. subtilis* DBC at 10^3 cfu/g (T4) during storage at 4°C.

CHAPTER 4

Influence of Proteolytic Spore Formers on Cottage Cheese Yield, Curd Size

Distribution, Physicochemical, Textural, and Sensory Attributes

INTRODUCTION

Spore formers are commonly found in dairy processing facilities. They are widely known for their spoilage-causing activities in milk and dairy products. Their spore-forming ability increases their survivability during heat treatment which leads to their presence in a final product. Most common spore-forming species found in dairy environments belong to the genus *Bacillus*. *Bacillus* can produce many diverse enzymes that can impact the texture of dairy products and produce typical off-flavors (Lücking et al., 2013). *Bacillus* spp. have the ability to produce many diverse enzymes, including proteases and lipases which can influence the value and quality of dairy products by affecting their sensory properties including texture, taste, aroma, and nutritional value (Teh et al., 2014). Researchers have observed that the proteolytic activity of *Bacillus* is associated with texture as well as flavor defects such as bitterness (Davies and Wilkinson, 1973; Frank, 1997). *Paenibacillus* spp. are also considered to be spoilage-causing flora in milk and dairy products. *Paenibacillus* spp. are predominantly psychrotolerant which can grow at refrigeration temperature (Ivy et al., 2012). Heyndrickx and Scheldeman (2002) concluded that although a low number of *Paenibacillus* spores can be found in raw and pasteurized milk, they have the potential to cause spoilage in refrigerated products during storage.

Fermentation is one of the most important steps in the manufacturing of cultured dairy products (Lucey, 2004). Any influence during fermentation affects textural, rheological, and physicochemical properties (Lucey and Singh, 1997; Lucey, 2002). During manufacture of cottage cheese, milk proteins play the important role during fermentation to develop its structure and functionality. Cottage cheese dressing is a major component of cottage cheese which is mixed to cottage cheese curd once it manufactured. The ratio of cottage cheese curd to dressing depends on the manufacturer (usually 1:1 ratio). Consequently, the quality of cottage cheese dressing is also as much as important as cottage cheese curd. Any contamination of proteolytic spore formers during manufacturing of cottage cheese curd or during the preparation of cottage cheese dressing may influence the final product quality.

The previous study had demonstrated that manufacturing of cottage cheese from the poor quality of raw milk could affect the finished product quality (Rash, 1990). Research study on somatic cells activity in milk and their influence on final cheese quality indicated that quality defects in cottage cheese by high somatic cell count in milk are likely mainly because of their proteolytic activity which resulted in a lower yield efficiency of cottage cheese (Klei et al., 1998). Cousin and Marth (1977) reported that skim milk inoculated with psychrotrophic bacteria before processing had increased non-protein nitrogen and non-casein nitrogen content of milk, whereas manufacturing time of cottage cheese made from that proteolyzed skim milk was found to be decreased. According to Mohamed and Bassette, (1979), the yield of cottage cheese found to be lower when cottage cheese was made from milk contaminated with the high number of psychrotrophs.

Many literatures had shown spoilage-causing activities in cottage cheese by various bacterial spp. but as per our knowledge, no research has been done which shows their influence during fermentation and if cottage cheese made from the contaminated cream dressing. The objective of this study was to determine the influence of proteolytic spore formers on cottage cheese properties in terms of yield, physicochemical, textural, and sensory attributes. In this study, we have inoculated proteolytic spore formers at the fermentation step during manufacturing of cottage cheese curd and during the preparation of cottage cheese cream dressing. The contaminated cottage cheese was evaluated for yield, curd size distribution, proteolysis, textural, sensory properties, and shelf life.

MATERIALS AND METHODS

Spore formers and Cottage Cheese Culture

Spore-forming strains, *B. subtilis* DBC, and *P. polymyxa* DBC1 were used in this study which was isolated from dairy environments and identified them up to their species level using the 16S rRNA gene sequencing technique. The original isolates were grown in Oxoid™ tryptone soya broth (Fisher Scientific, Pittsburgh, PA, USA) and incubated overnight at 37°C in the case of *B. subtilis* DBC, whereas *P. polymyxa* DBC1 was incubated at 30°C. Isolates were preserved for future use as described by Perry (1995). The actively growing isolates were pelleted out by centrifugation (Benchtop Refrigerated Centrifuge, Model: CR 412, Jouan In., VA, USA) at 3000 rpm for 10 min. Cell pellets were suspended in 0.01 M phosphate-buffered saline (PBS; Fisher Scientific, Pittsburgh, PA, USA) at pH 7.4 and preserved in 1.8 mL glycerol cryo-vials (Crockbank™, Copan

diagnostic Inc., CA, USA) containing sterile beads. All isolates cryo-vials were stored at -75°C in a deep freezer (Ultralow Freezer, NUAIRE Inc., MN, USA).

Isolates were identified as a spore former by sporulating them as per the method suggested by Novak et al. (2005) and Martinez et al. (2017) with some modification. A sterile bead of each isolate was added to 10 mL of Oxoid™ tryptone soya broth and incubated at 37°C for *Bacillus subtilis* DBC, and 30°C for *P. polymyxa* DBC1 up to 5 days and checked for the presence of spores during incubation by spore staining method (Hamouda et al., 2002). Both isolates, *B. subtilis* DBC, and *P. polymyxa* DBC1, were sporulated during incubation which confirmed that selected *Bacillus* spp. were spore formers.

For the activation of spore formers from frozen cryo-vials, a sterile bead of each isolate was transferred into Oxoid™ tryptone soya broth (Fisher Scientific, Pittsburgh, PA, USA) and incubated overnight at 37°C for *Bacillus subtilis* DBC and 30°C for *P. polymyxa* DBC1. This culture activation step was performed three times with 0.1% (w/w) inoculation. At the third step of activation, cells at the mid-exponential phase of each isolate were pelleted out by centrifugation at 3000 rpm for 10 min. The pellets were suspended into 0.01 M phosphate-buffered saline (PBS; Fisher Scientific, Pittsburgh, PA, USA) and cells were washed by vortexing for 5 min, followed by centrifugation (Benchtop Refrigerated Centrifuge, Model: CR 412, Jouan In., VA, USA) at 3000 rpm for 10 min. Washed cells pellets of isolates were resuspended again in PBS and used them as a working culture.

Selected spore formers, *B. subtilis* DBC, and *P. polymyxa* DBC1, were characterized as proteolytic strains based on a skim milk agar method (Jones et al., 2007).

Commercial cottage cheese culture (Chr. Hansen, Milwaukee, WI, USA), F DVS R-604 was used as the acidifying culture at the rate of 0.01% (w/w) for the manufacturing of cottage cheese.

Psychrotrophic Proteolytic Behavior of *P. polymyxa* DBC1

For the identification of *P. polymyxa* DBC1 as a psychrotrophic proteolytic strain, *P. polymyxa* DBC1 was inoculated at approximately 10^5 cfu/mL from the exponential phase in a dispersion of heat treated non-fat dry milk (11%, w/v) and incubated at 4°C for 10 days along with a control (without added with *P. polymyxa* DBC1). Non-protein nitrogen (NPN% \times 6.38) content (Hooi et al. (2004) of milk inoculated with *P. polymyxa* DBC1 and control was evaluated after 10 days of storage. NPN content of milk inoculated with *P. polymyxa* DBC1 (%NPN \times 6.38: 0.278) was found to be higher as compared to control milk (% NPN \times 6.38: 0.174) which indicates that *P. polymyxa* DBC1 is a psychrotrophic proteolytic spore former.

Experiment Design

The experimental block consists of a total of 5 treatments (Table 1) from the same skim milk and cream dressing. Curd produced from skim milk without added spore former was mixed with cream dressing (without added spore former) was the treatment T1. In the treatment (T2), cottage cheese curd was taken from T1 treatment and mixed with cream dressing which was inoculated with psychrotrophic proteolytic *P. polymyxa* DBC1 (approximately 10^5 cfu/mL). In treatment T3, curd produced from skim milk added with rennet mixed with cream dressing (without added spore former). In the treatment 4, curd produced from skim milk inoculated with *B. subtilis* DBC was mixed

with cream dressing (without added spore former) and in the treatment 5, curd produced from skim milk inoculate with a cocktail of *B. subtilis* DBC and *P. polymyxa* DBC1 was mixed with cream dressing (without added spore former). Cottage cheese curd from each treatment (T1, T3, T4, and T5) was weighed to calculate the yield. The weighed curd was blended in a ratio of 1 to 1 with cream dressing (8% fat) and kept it for 15 min to allow absorption of cream dressing by the curd. The creamed cottage cheese was packaged in 16 oz. plastic containers and stored at 4°C for the shelf life study. This experiment was replicated three times.

Cream Dressing Preparation

A batch of 30 Kg of cream dressing (8% fat) was prepared by procuring a cream (35% fat) and skim milk from the Davis dairy plant facilities at South Dakota State University. Salt (Top-Flo Granulated Salt, Cargill, MN, USA) was added at the rate of 1.65% (w/w) to the cream dressing. The standardized cream dressing was heat treated to 74°C for 30 min, followed by cooling to 60°C and homogenized (1st stage: 2000 psi, 2nd stage: 500 psi). The cream dressing was cooled and adjusted to a pH of 5.7 using lactic acid (Across Organics™, 85% DL-lactic acid, Fisher Scientific, Pittsburgh, PA, USA).

T1: Cottage Cheese Making Without Rennet and Without Added any Spore Former

Skim milk (approximately 90 kg) was procured from the Davis dairy plant facilities at South Dakota State University. Skim milk was pasteurized at 72°C for 16 s and transferred to a cheese vat (Kusel Equipment Co., Watertown, WI). Pasteurized skim milk was set at 30°C and inoculated with cottage cheese culture F DVS R-604 at the rate of 0.01% (w/w). Inoculated milk (30°C) was allowed to coagulate to a pH approximately

4.6 until the curd firm enough, followed by cutting of curd based on the curd firmness using the 1/4-inch wire knives (horizontal, vertical, and cross cut vertical) and after cutting, the curd was allowed the curd to heal for 30 min. A quarter of the whey was removed from a cheese vat and heated to 55°C in a steam chest. This heated whey was then added back to the vat and gently stirred for 5 minutes. This process was repeated for an hour until the vat temperature reached 46°C. Once the vat temperature reached 46°C, a quarter of the whey was removed from a cheese vat and heated to 63°C in the steam chest. This heated whey was added back to the vat and gently stirred for 5 minutes. This process was repeated for an hour until the vat temperature reached 52°C. After cooking the curd, whey was drained from the vat, and the curd was washed first time using chilled water and agitated for 5 min, followed by draining of washed water. In the second time, the curd was washed using chilled water and ice and agitated until the ice had melted completely, followed by draining of washed water. The quantity of cottage cheese curd was weighed for the calculation of the yield. The weighed curd was blended in a ratio of 1 to 1 with cream dressing and kept for 15 min to allow absorption of cream dressing by the curd. The creamed cottage cheese was packaged in 16 oz. plastic containers covered with lids and stored at 4°C for the shelf life study. This served as the treatment T1.

T2: Cottage Cheese Curd and Cream Dressing Added with a Psychrotrophic Proteolytic Spore Former

The cream dressing was inoculated with psychrotrophic proteolytic *P. polymyxa* DBC1 at the level of approximately 10^5 cfu/mL from the exponential phase and mixed in a ratio of 1:1 to a portion of approximately 5 Kg taken out from the treatment T1.

Creamed cottage cheese was packaged in 16 oz. plastic containers covered with lids and stored at 4°C for the shelf life study. This served as the treatment T2.

T3: Cottage Cheese Making with Rennet but Without Added any Spore Former

For the preparation of cottage cheese with rennet (treatment 3), the same method was followed as used for the treatment T1 except for rennet addition step in milk. When milk reached to a pH 6.2 during fermentation, rennet was added at the rate of 0.84 mL per 1000 Kg of milk. Rennet (CHY-MAX[®] Extra, Chr. Hansen, Milwaukee, WI, USA) was diluted in a ratio of 1 to 40 with distilled water. This served as the treatment T3.

T4 and T5: Cottage Cheese Making from Milk Added with Proteolytic Spore Formers

Proteolytic spore formers were inoculated at the level of approximately $10^5 - 10^6$ cfu/mL from their exponential phase when the milk was inoculated with cottage cheese culture. Afterward, the same protocol was followed as the treatment T1 for cottage cheese preparation. Cottage cheese made from milk added with a spore former, *B. subtilis* DBC and a cocktail of spore formers, *B. subtilis* DBC and *P. polymyxa* DBC1 were considered as the treatments T4 and T5 respectively. The weighed curd from each treatment was blended in a ratio of 1 to 1 with cream dressing and kept for 15 min to allow absorption of cream dressing by the curd. The creamed cottage cheese was packaged in 16 oz. plastic containers covered with lids and stored at 4°C for the shelf life study.

Analysis

The pH of milk, whey, cottage cheese curd, and the cream dressing was measured using a pH meter (Orion StarTM A421 pH meter, Fisher Scientific, Pittsburgh, PA, USA). Skim milk was analyzed for total nitrogen and non-casein nitrogen content using the Kjeldahl method (Hooi et al., 2004) to calculate casein nitrogen of skim milk. Fat content of cream and cream dressing was analyzed using the Mojonnier extraction method (Atherton and Newlander, 1977). Total solids (TS) content of cottage cheese curd was analyzed by drying 2 g of cottage cheese curd for 24 h in a forced-draft oven (Lindberg/Blue MTM, Asheville, NC, USA) set at 100°C (AOAC, 1995).

Gel Strength and Microstructure Evaluation of Set Curd

During preparation of cottage cheese for T1, T3, T4, and T5 treatments, thirty mL of milk was transferred to a 50 mL of glass beaker from a cheese vat after the starter culture inoculation step (T1) and after the starter culture and spore formers inoculation step (T4 and T5), whereas for treatment T3, milk was transferred after renneting and incubated in a water bath at 30°C to simulate the milk in the cheese vat for each treatment. Once the curd was set, beakers containing milk with the respective treatments were analyzed for gel strength and microstructure of set curd.

A stress-controlled rheometer (ATS Rheosystems, Rheologica Instrument Inc., Bordentown, NJ, USA) was used to perform the frequency sweep. A plate and plate assembly (diameter 30 mm) with a gap of 2 mm were used for the dynamic oscillatory measurement. Fine sandpaper (400 grit, Wetordry, 3M) was cut and glued to the upper plate of the rheometer to prevent sample slippage. The frequency dependency on the

elastic modulus of set curd was determined by first conducting a stress sweep measurement at a constant frequency of 0.15 Hz. The frequency sweep was performed by subjecting an undisturbed piece of set curd of 1.6 g at a constant stress of 1 Pa, with frequency ranging from 0.1 to 1 Hz in 10 steps. The gel strength of the set curd was measured by evaluating the elastic modulus (G' , Pa) at 1 Hz at 4°C.

The method of Meletharayil et al. (2015) was used to study the microstructure of set curd by performing the confocal laser scanning microscopy (CLSM) with some modification. One g of undisturbed set curd was stained with 100 μ L of 0.2% (w/w) acridine orange (Sigma Chemical Co., St. Louis, MO, USA) which will stain proteins. The Olympus FV 1200 Fluoview confocal scanning laser microscope (Olympus, Tokyo, Japan) with an air-cooled Ar/Kr laser with an excitation of 488 nm was used to evaluate the microstructure cottage cheese using Olympus LUCPLFLN 40x objective lens with 0.6 numerical aperture. The captured images were analyzed as a grayscale 8-bit image using ImageJ software (US National Institutes of Health, Bethesda, MD, USA).

Curd Size Distribution and Yield

Curd size distribution analysis of manufactured cottage curd was performed as per the method described by Kosikowski (1963) with some modification. Approximately 500 g of un-creamed curd was mixed with 2 L chilled water, followed by gentle agitation for 1 min. The mixture of curd and water was layered over the stacked metal sieves (Fisher Brand Test Sieve, Fisher Scientific, Pittsburgh, PA, USA) immersed in water with 12.5 mm, 8 mm, 4 mm, and 1 mm wire mesh from top to bottom. The stack of sieves was vigorously shaken for 30 s. Each sieve with curd is separately weighed, and the weight of

curd on each sieve was calculated. The curd size distribution on each sieve was reported as a percentage of the total weight of curd on all the sieves. The percentage of curd particle on the smallest mesh sieve, 1 mm sieve, is called "grit" or cheese dust. It represents the most important value within the distribution pattern since it reflects shattered curd particles produced during manufacture. The example images of stacked metal sieves immersed in a water and cottage cheese curd on each sieve are shown in Figure 1.

Proteolysis Measurement

Creamed cottage cheese was liquified at high speed using Osterizer® 12-Speed Blender by grinding for 2 min. The cream cottage cheese slurry was analyzed for non-protein nitrogen [$\% \text{NPN} \times 6.38$] content as a measure of proteolysis described by Hooi et al., (2004). A sample size of 1.5 g was used, and the NPN content of cottage cheese was measured on the 1st, 14th, and 28th day of storage.

The same cottage cheese slurry was dissolved in a ratio of 1 to 2 with cheese extraction buffer as per method described by Biswas (2016). A dispersion was kept for 6 h at 4°C to dissolve cottage cheese slurry and transferred a small quantity of it in 1 mL Eppendorf tube, followed by centrifugation (Eppendorf 5415D centrifuge, Eppendorf, Hauppauge, NY, USA) at 10000 rpm for 15 min for the fat removal. Defatted dispersion of creamed cottage cheese was analyzed further for the individual protein fractions present in creamed cottage cheese using capillary gel electrophoresis (CE) as per method described by Salunke (2013) and Biswas (2016). Peaks of individual protein fractions on CE electrophoretogram for each creamed cottage cheese sample including low molecular

weight peptides (LMW), other peptides (OP), α -lactalbumin (α -LAC), β -lactoglobulin (β -LG), β -casein (β -CN), γ -casein (γ -CN), α S1 casein (α S₁-CN), α S₂ casein (α S₁-CN), and κ -casein (κ -CN) were identified, and the area of each identified peak was calculated as percentage of total area of identified positive peaks according to Salunke (2013) and Biswas (2016). The level of proteolysis of creamed cottage cheese was indicated by calculating LMW, and OP identified on CE electrophoretogram of that respective creamed cottage cheese sample. LMW and OP are produced from the hydrolysis of serum protein and casein present in creamed cottage cheese. Higher the values of LMW and OP are indicating more proteolysis induced in creamed cottage cheese. Creamed cottage cheese was evaluated for the individual protein fractions present in creamed cottage cheese using CE on 1st, 14th, and 28th day of storage.

Texture Analysis

The firmness of cottage cheese was measured by a large deformation penetration test using TA-XTplus Texture Analyzer (Texture Technologies Corp., South Hamilton, MA, USA) as per method described by Meletharayil et al., (2016). Creamed cottage cheese was transferred on a 1 mm wire mesh size (Fisher Brand Test Sieve, Fisher Scientific, Pittsburgh, PA, USA) and allowed cream dressing to be drained for 15 min at 4°C. A 30 g sample of the drained curd was transferred in a plastic container with 43.8 mm diameter and tapped 10 times to avoid gaps between the curd particles in a container. The firmness of cheese was performed using a 1.5-inch diameter cylindrical probe with a test speed and post-test speed of 0.5 mm. s⁻¹ to the distance of 10 mm using a 5-Kg load cell. Firmness was defined as the total positive area derived from the force-distance

curve. Cottage cheese curd was analyzed for firmness on 1, 14, and 28 days of storage at 4°C.

Sensory Analysis

Cottage cheese was evaluated for descriptive sensory analysis of flavor and texture such as bitterness and firmness at North Carolina State University (Department of Food, Bioprocessing and Nutrition Sciences, NCSU, Raleigh, NC). Cottage cheeses were dispensed into coded cups approximately 30 min before each panel. Descriptive analysis was performed on a 0 to 15-point universal intensity scale with the SpectrumTM method (Meilgaard and others 1999; Drake and Civille 2003; Drake, 2004, Drake et al., 2009). A descriptive sensory panel (n=7, 5 females, 2 males, ages 22 – 46 y) with more than 200 h experience with the descriptive analysis of cottage cheese flavor and texture evaluated the cottage cheese samples. Cottage cheese was shipped for sensory evaluation within a week of manufacture and at the end of shelf life (between 25 to 28 days of storage at 4°C).

Statistical Analysis

Results were analyzed by ANOVA using R-3.4.0 software (R Core Team, Vienna, Austria) and significant results were indicated by $P < 0.05$. LSD test was used to determine the significant difference between the means. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

pH, Fermentation Time, and Gel Strength

The pH, fermentation time, and gel strength were monitored at a set of curd just prior to cutting during the manufacture of cottage cheese (T1, T3, T4, and T5) shown in Table 2. The cutting step was decided based on the curd firmness by checking it using a cheese knife, not based on the final pH value. The fermentation time was indicated as the total time is required from the addition of starter culture to milk until the time of the curd cutting step. The fermentation time was observed to be higher for cottage cheese made with the treatment T1 with a lower pH value at the time of cutting whereas the fermentation time was observed to be lower for rennet (T3) and proteolytic strains (T4 and T5) added cottage cheese with a higher pH value at the time of cutting. Gel strength of curd was monitored at the cutting stage. A higher gel strength was observed for T3, T4, and T5 cottage cheese with the highest value of 19.2 Pa for rennet added cottage cheese (T3) of set curd while a lower gel strength (G' :12.3 Pa) was observed for cottage cheese without added any rennet and proteolytic strains (T1). Results for rennet added cottage cheese can be compared with rennet-acid induced milk gel whereas the results of cottage cheese with the treatment T1 can be compared with acid-induced milk gel. Lucey et al., (2000) reported that milk gel formed by rennet acid coagulation had high viscoelastic properties when compared to only acid induced milk gel. The combined effect of rennet-induced κ -CN hydrolysis and acid-induced gelation by added starter culture might have and formed a compact protein network by protein-protein interactions and hence, observed with the highest gel strength of curd. Cousin and Marth (1997) observed a decrease in time to set the curd and increased curd firmness in cottage cheese

made from skim milk which had been inoculated with psychrotrophic bacteria before processing. As we discussed in our previous study on sour cream, the limited level of proteolysis induced by proteolytic spore-forming *Bacillus* spp. might have resulted in inter- and intra- molecular interactions of protein which may resulted in a dense protein network in *Bacillus* added sour cream. Same interpretations can be expected for cottage cheese added with proteolytic spore formers (T4 and T5). As shown in Fig. 3, microstructure evaluation of cottage cheese curd also revealed the compact protein network in rennet (T3) and proteolytic strains (T4 and T5) added cottage chase as compared to the treatment T1. There was a significant impact of a cocktail of spore formers (T5) was observed on a gel strength of curd and fermentation time as compared to treatment T1.

Curd Size Distribution and Yield of Cottage Cheese

Curd size distribution on each sieve was reported as the percentage of the total weight of curd on the four sieves shown in Table 3 for cottage cheese with different treatments. Curd particles on 12.5 and 8 mm sieves indicate matting of curd whereas small curd particles on 1 mm sieve are called “grit.” Matting of curd and grit are undesirable for cottage cheese industry as it is associated with the poor manufacturing quality. A high grit indicates curd shattering and can reduce the yield (Cross et al., 1977). Curd particles on 4 mm sieve can be considered as the ideal curd. Results demonstrated that cottage cheese manufactured with proteolytic spore formers (T4 and T5) had significant ($P < 0.05$) higher grit values with the grit of 19.3% for cottage cheese with the treatment T4 whereas rennet added cottage cheese (T3) had the lowest grit value. This is due to the more compact structure of cottage cheese curd when rennet is added (Fig. 1B)

which reduces the shattering of curd during manufacturing. In contrast, when uneven aggregated structures are formed during coagulation with added proteolytic spore formers (Fig. 1C and 1D) resulted in a brittle curd and hence, produced more grit upon shattering during cottage cheese manufacture. Matting of curd was observed in cottage cheese with all treatments, and the higher value was observed for rennet added cottage cheese (T3). The highest ($P < 0.05$) ideal curd particle size was observed for rennet added cottage cheese (T3). Curd size distribution analysis can be correlated with the yield of cottage cheese (Table 3). Cottage cheese manufactured with proteolytic strains showed higher grit values which significantly ($P < 0.05$) reduced the yield of cottage cheese (T4:13.10%, T5:14.30%) as compared to treatments T2 and T1.

Proteolysis

The non-protein nitrogen [NPN (%)] content of cottage cheese for each treatment is shown in Fig. 2. Cottage cheese mixed with a psychrotrophic proteolytic spore-former added cream dressing (T2), cottage cheese added with a cocktail of proteolytic strains (T5), and rennet added cottage cheese (T3) showed significantly ($P < 0.05$) higher NPN values on the 1st day of storage. This is due to proteolytic activity from the added rennet and spore formers during cheese making. Rennet added cottage cheese (T3) was observed with the highest NPN content ($P < 0.05$) throughout the storage as compared to other treatments, primarily due to hydrolysis of κ -CN. Storage study of cottage cheese revealed that NPN content of cottage cheese for all treatments were increased throughout the storage. This could be due to the proteolytic activity of starter culture, added rennet and spore formers during storage.

The individual protein fractions present in cottage cheese quantified by capillary gel electrophoresis (CE) are shown in Table 4. The percentage area of low molecular weight peptides (LMW) and other peptides (OP) was higher for rennet added cottage cheese (T3), primarily due to hydrolysis of κ -CN. Results demonstrated that cottage cheese T2, T3, T4, and T5 contained γ -CN which indicates hydrolysis of β -CN. The value for γ -CN was higher for cottage cheese added with proteolytic strains (T2, T4, and T5). During storage, the level of proteolysis increased in all treatments. These findings are in agreement with the NPN results.

Texture Profile Analysis

The firmness of cottage cheese was determined at 1, 14, and 28 days of storage shown in Table 5. Cottage cheese added with rennet (T3) and proteolytic strains (T4, and T5) had higher firmness on the 1st day of storage as compared to T1. Rennet added cottage cheese had observed with the highest ($P < 0.05$) firmness as compared to all other treatments. No significant ($P > 0.05$) difference was observed for cottage cheese added with a cocktail of proteolytic strains (T5) as compared to single proteolytic strain added cottage cheese (T4). A higher firmness of cottage cheese for T3, T4, and T5 may be due to their higher gel strength at the set (Table 2). As expected T1 and T2 showed a similar trend of firmness since a portion of curd from T1 was used for T2. There was no synergetic growth impact of proteolytic spore formers on the firmness of cottage cheese. Refrigerated storage significantly ($P < 0.05$) increased the firmness of cottage cheese with all treatments (T1, T2, T3, T4, and T5). Firmness of cottage cheese was increased over a period of storage. The value of firmness for all cottage cheese was increased significantly ($P < 0.05$) from 1st day to 14th day of storage, consequently no significant (P

> 0.05) change in firmness was observed between 14 and 28 days stored cottage cheese. Higher firmness values for rennet added cottage cheese (T3) and proteolytic strains added cottage cheese (T4 and T5) can be correlated with the results obtained from their microstructure evaluation (Fig. 3). As shown in figure 3, the aggregated protein network was produced during coagulation of cottage cheese made with rennet (Fig. 3B), and proteolytic spore formers (Fig. 3C and 3D) as compared to T1 (Fig. 3A).

Sensory Evaluation

Cottage cheese samples were evaluated for descriptive flavor and texture profiles by trained sensory panelists using a universal intensity scale from 0 to 15 by spectrum method (Meilgaard et al., 1999). Sensory results indicated the development of bitter taste during storage (Fig. 4 A). On the 1st week of storage, there wasn't any sign of bitterness in any of the cottage cheese samples, but at the end of storage (last week), bitterness was developed in all cottage cheese samples. The level of bitterness was higher in T2, T3, T4, and T5 cottage cheese samples with the highest bitterness observed in T4. Sensory evaluation was performed in a triplicate on cottage cheese samples, and only one replicate of T1 cottage cheese was bitter whereas rennet (T3) and proteolytic strains added samples (T2, T4, and T5) had bitterness in all replicates. This indicates that proteolytic activity during storage in rennet (T3) and proteolytic strains (T2, T4, and T5) added cottage cheese samples can result in the development of a bitter taste at the end of storage. NPN results indicated higher proteolytic activity in rennet and proteolytic strains added cottage cheese. Capillary gel electrophoresis results had shown the presence of γ -CN in rennet and proteolytic strains added cottage cheese which indicates the hydrolysis of β -CN. Previous researchers reported that bitter peptides are released from casein

primarily by the action of rennet and bacterial proteinases (Sullivan and Jago, 1972; Sullivan et al., 1973). β -CN could be the source of bitter peptides produced by the proteinase, while chymosin (rennet) may produce bitter peptides from all casein components (Sullivan and Jago, 1972; Exterkate, 1976; Exterkate, 1983). These findings explain the development of bitterness in cottage cheese samples. The firmness of cottage cheese by sensory evaluation (Fig. 4B) can be correlated with firmness results obtained from texture profile analysis (Table 5). Rennet added cottage cheese had the highest firmness among all treatments, and the value of firmness increased during storage for all treatments.

CONCLUSION

Cottage cheese made from milk inoculated with selected proteolytic spore-formers at 10^5 cfu/mL prior to fermentation had influenced cottage yield, physicochemical, textural, and sensory properties as compared to cottage cheese added with rennet (T3) and without rennet (T1). Lower fermentation time and a higher pH were observed for proteolytic strains and rennet added cottage cheese. The grit value was observed highest for cottage cheese added with proteolytic-spore formers (T4 and T5), therefore resulted in a lower yield. Cottage cheese made from milk inoculated with selected proteolytic spore-formers had brought textural and sensory changes as compared to cottage cheese added with rennet (T3) and without rennet (T1). Negative impact on sensory had observed for cottage cheese mixed with a psychrotrophic proteolytic spore-forming strain contaminated cream dressing without impact on texture. A higher firmness was observed for rennet, and proteolytic strains added cottage cheese and observed for bitterness at the end of shelf life. Bitterness was observed for cottage cheese mixed with a

psychrotrophic proteolytic spore-forming strain added cream dressing at the end of the shelf life, but it didn't have any influence on cottage cheese texture. Microstructure evaluation of cottage cheese curd indicated the aggregated protein network in proteolytic strains added cottage cheese (T4 and T5) which found to be different structure as compared to rennet (T1) and without rennet (T3) added cottage cheese. Capillary gel electrophoresis results indicated the hydrolysis of β -CN by the development of γ -CN, and NPN indicated proteolysis which could be associated with the bitterness in cottage cheese during storage. Refrigerated storage had an impact on the firmness of cottage cheese for all treatments resulted in the increased firmness during storage. Development of bitterness at the end of shelf life probably because of the continuation of proteolytic activity even during storage. The present work indicates that cottage cheese made from milk contaminated with proteolytic spore-forming strains can negatively impact on yield, physicochemical, textural, and sensory properties, and the shelf life. Strategies to control spore formers in cottage cheese need to be developed.

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Table 1. Design of experiment consisting a total of five cottage cheese treatments

Treatments				
T1	T2	T3	T4	T5
Cottage cheese was prepared without rennet and without any spore former	Some quantity of cottage cheese curd was taken out from T1 treatment and mixed with cream dressing inoculated with psychrotrophic proteolytic <i>P. polymyxa</i> DBC1 (approximately 10^5 cfu/mL)	Cottage cheese was prepared with rennet but without any spore former	Cottage cheese was prepared without rennet but added with a proteolytic spore former, <i>Bacillus subtilis</i> DBC (approximately 10^5 cfu/mL)	Cottage cheese was prepared without rennet but added with a cocktail of proteolytic spore formers, <i>Bacillus subtilis</i> DBC and <i>P. polymyxa</i> DBC1 (approximately a total of 10^5 cfu/mL)

Table 2. pH, fermentation time, and gel strength of cottage cheese

Treatments ³	pH ^{1,2}	Fermentation time (min) ^{1,2}	Gel strength (G', Pa) ^{1,2}
T1	4.61	441	12.3
T3	4.85	385	19.2
T4	4.69	423	15.9
T5	4.77	408	16.2

¹ n=3² Values were monitored at a set of curd just prior to cutting³T1: Cottage cheese made without added any spore formers and rennet, T3: Rennet added cottage cheese but without added any spore formers, T4: *B. subtilis* DBC added cottage cheese, T5: a cocktail of *B. subtilis* DBC and *P. polymyxa* DBC1 added cottage cheese

Table 3. Curd size distribution and yield of cottage cheese

Treatments ³	Sieves (mm) ^{1,2}				% Yield ²
	12.5	8	4	1	
T1	2.9 ^b	7.8 ^a	79.9 ^b	9.4 ^c	15.10 ^a
T3	4.4 ^a	7.4 ^a	82.6 ^a	5.6 ^d	15.00 ^a
T4	2.4 ^{bc}	5.6 ^b	72.8 ^d	19.3 ^a	13.10 ^c
T5	1.9 ^c	7.6 ^a	74.3 ^c	16.2 ^b	14.30 ^{bc}

^{a-c} Means of treatments in the same column without common superscripts are significantly different ($P < 0.05$)

¹Curd size distribution on each sieve was reported as a percentage of the total weight of curd on the four sieves

²n=3

³T1: Cottage cheese made without added any spore formers and rennet, T3: Rennet added cottage cheese but without added any spore formers, T4: *B. subtilis* DBC added cottage cheese, T5: a cocktail of *B. subtilis* DBC and *P. polymyxa* DBC1 added cottage cheese

Table 4: Protein fractions (%) present in cottage cheese observed on capillary gel electrophoresis electrophoretogram

Time interval (days)	Treatments ²	Protein fractions (%) ¹							
		LMW+OP	α -LAC	β -LG	β -CN	γ -CN	α S ₁ -CN	α S ₂ -CN	κ -CN
1 st	T1	2.36	0.74	1.68	38.85	0.00	38.77	14.07	6.53
	T2	2.38	0.79	2.01	39.44	0.92	36.38	12.80	5.28
	T3	6.50	0.82	1.64	37.15	0.70	37.71	12.90	0.57
	T4	2.34	0.78	1.62	35.07	1.10	38.57	14.67	5.87
	T5	2.77	0.82	2.13	37.54	1.02	37.65	12.65	5.44
14 th	T1	2.51	1.08	1.78	37.48	0.00	38.62	12.19	5.98
	T2	3.50	0.88	1.80	37.45	0.33	38.35	11.82	5.31
	T3	7.82	0.90	1.92	37.16	0.94	37.27	10.98	0.72
	T4	2.47	0.79	1.91	39.28	0.40	40.20	10.72	4.64
	T5	2.98	0.95	1.92	37.29	0.28	40.62	11.77	4.18
28 th	T1	2.63	0.85	1.81	36.83	0.00	38.55	12.81	6.72
	T2	3.66	1.05	2.00	36.69	0.61	38.72	12.10	5.37
	T3	8.14	0.91	1.86	36.44	0.46	38.12	11.62	0.37
	T4	2.82	0.89	1.68	35.42	1.02	38.68	12.84	7.08
	T5	3.77	1.14	2.21	35.31	1.04	35.31	12.41	6.38

¹ n=3

LMW: Low molecular weight peptides, OP: Other peptides, α -LAC: α -lactalbumin, β -LG: β -lactoglobulin, β -CN: β -casein, γ -CN: γ -casein, α S₁-CN: α S₁ casein, α S₂-CN: α S₂-casein, κ -CN: κ -casein

²T1: Cottage cheese made without added any spore formers and rennet, T2: Cottage cheese mixed with the cream dressing added with proteolytic spore former, T3: Rennet added cottage cheese but without added any spore formers, T4: *B. subtilis* DBC added cottage cheese, T5: a cocktail of *B. subtilis* DBC and *P. polymyxa* DBC1 added cottage cheese

Table 5. Texture analysis of cottage cheese

	Time interval (days)	Firmness of cottage cheese during storage ¹				
		T1	T2	T3	T4	T5
Firmness (Kg·s)	1 st	1.27 ^{Cc}	1.31 ^{Bb}	1.86 ^{Ba}	1.33 ^{Bb}	1.38 ^{Bb}
	14 th	2.85 ^{ABbc}	2.47 ^{ABd}	3.12 ^{ABab}	2.61 ^{ABcd}	2.95 ^{ABbc}
	28 th	4.17 ^{Ac}	3.98 ^{Ac}	4.79 ^{Abc}	5.09 ^{Aa}	4.54 ^{Abc}

^{A-C} Means of treatments in the same column between the days or ^{a-d} Means of treatments in the same row on the same day without common superscripts are significantly different ($P < 0.05$)

¹n=6

T1: Cottage cheese made without added any spore formers and rennet, T2: Cottage cheese mixed with the cream dressing added with proteolytic spore former, T3: Rennet added cottage cheese but without added any spore formers, T4: *B. subtilis* DBC added cottage cheese, T5: a cocktail of *B. subtilis* DBC and *P. polymyxa* DBC1 added cottage cheese

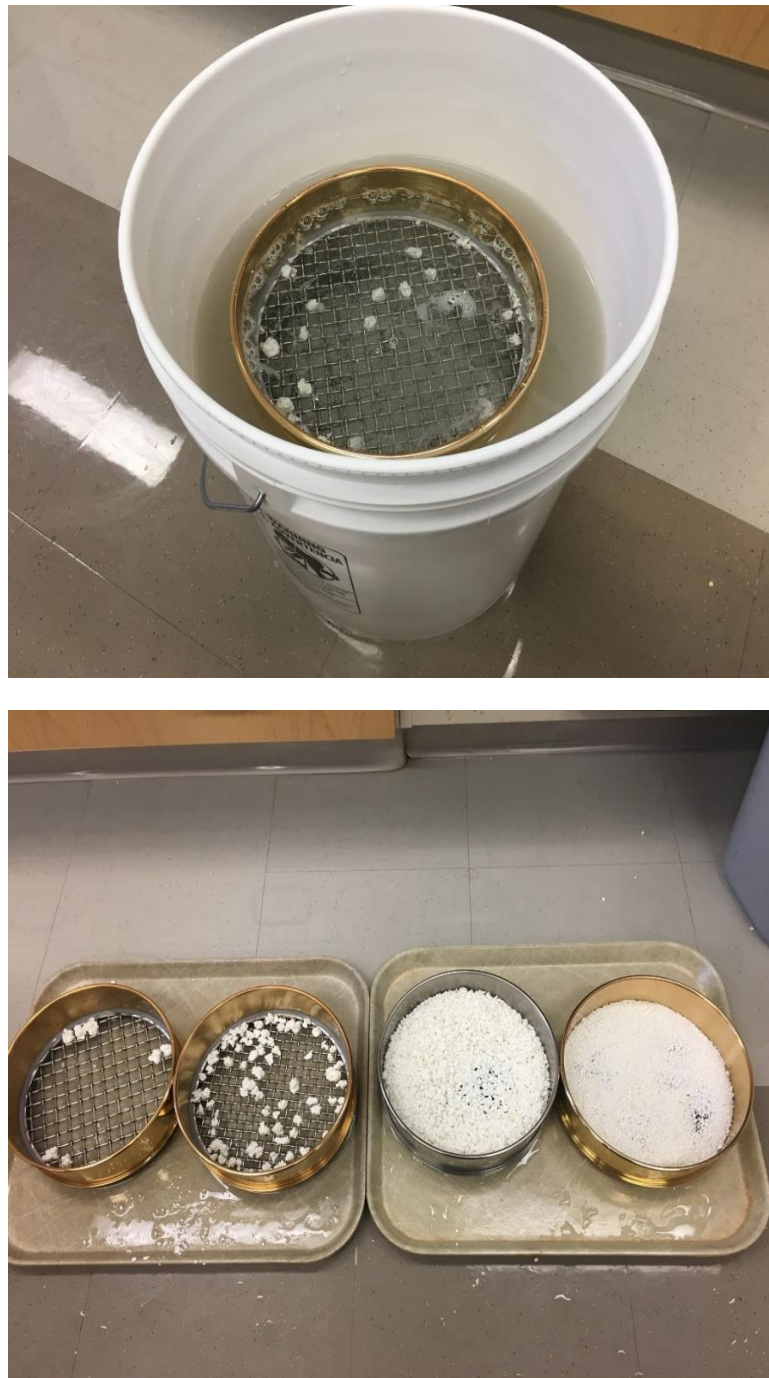


Figure 1. The example images of stacked metal sieves immersed in a water and distributed cottage cheese curd on each sieve for curd size distribution analysis

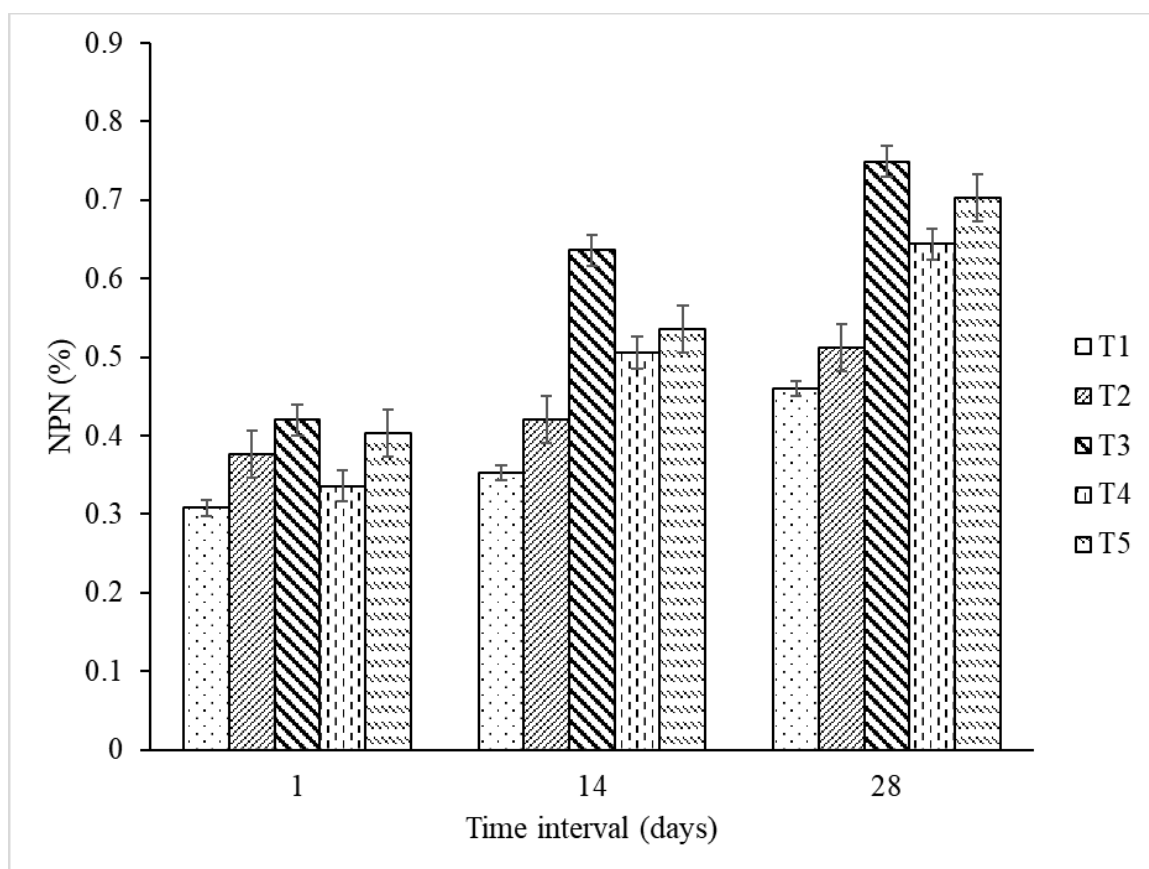


Figure 2. NPN (%; $N \times 6.38$) content of cottage cheese on 1, 14, and 28 days of storage at 4°C. T1: Cottage cheese made without added any spore formers and rennet, T2: Cottage cheese mixed with the cream dressing added with proteolytic spore former, T3: Rennet added cottage cheese but without added any spore formers, T4: *B. subtilis* DBC added cottage cheese, T5: a cocktail of *B. subtilis* DBC and *P. polymyxa* DBC1 added cottage cheese. $n=6$.

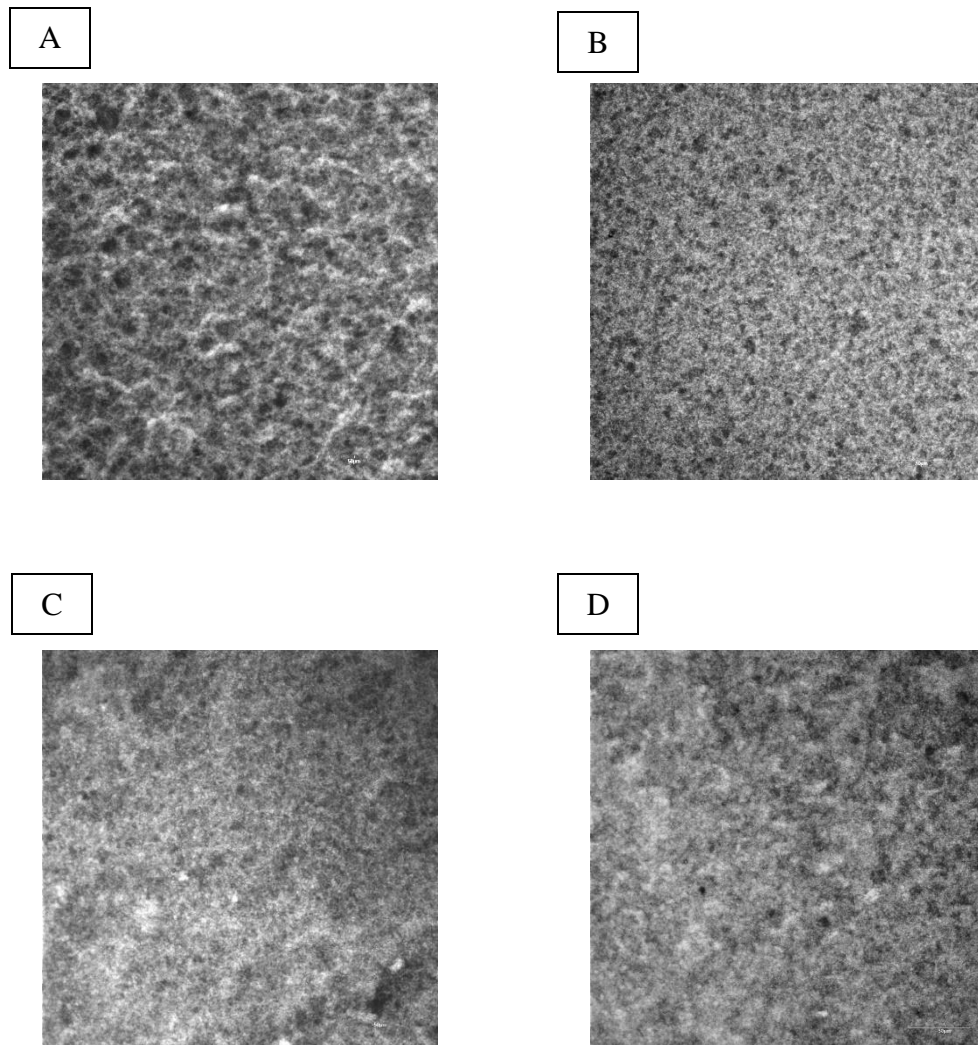


Figure 3. Microstructure evaluation of cottage cheese using confocal microscopy with Olympus LUCPLFLN 40x objective lens and 0.6 numerical aperture. Image A indicates treatment T1, Image B indicates treatment T3, Image C indicates treatment T4 and Image D indicates treatment T5. T1: Cottage cheese made without added any spore formers and rennet, T2: Cottage cheese mixed with the cream dressing added with proteolytic spore former, T3: Rennet added cottage cheese but without added any spore formers, T4: *B. subtilis* DBC added cottage cheese, T5: a cocktail of *B. subtilis* DBC and *P. polymyxa* DBC1 added cottage cheese

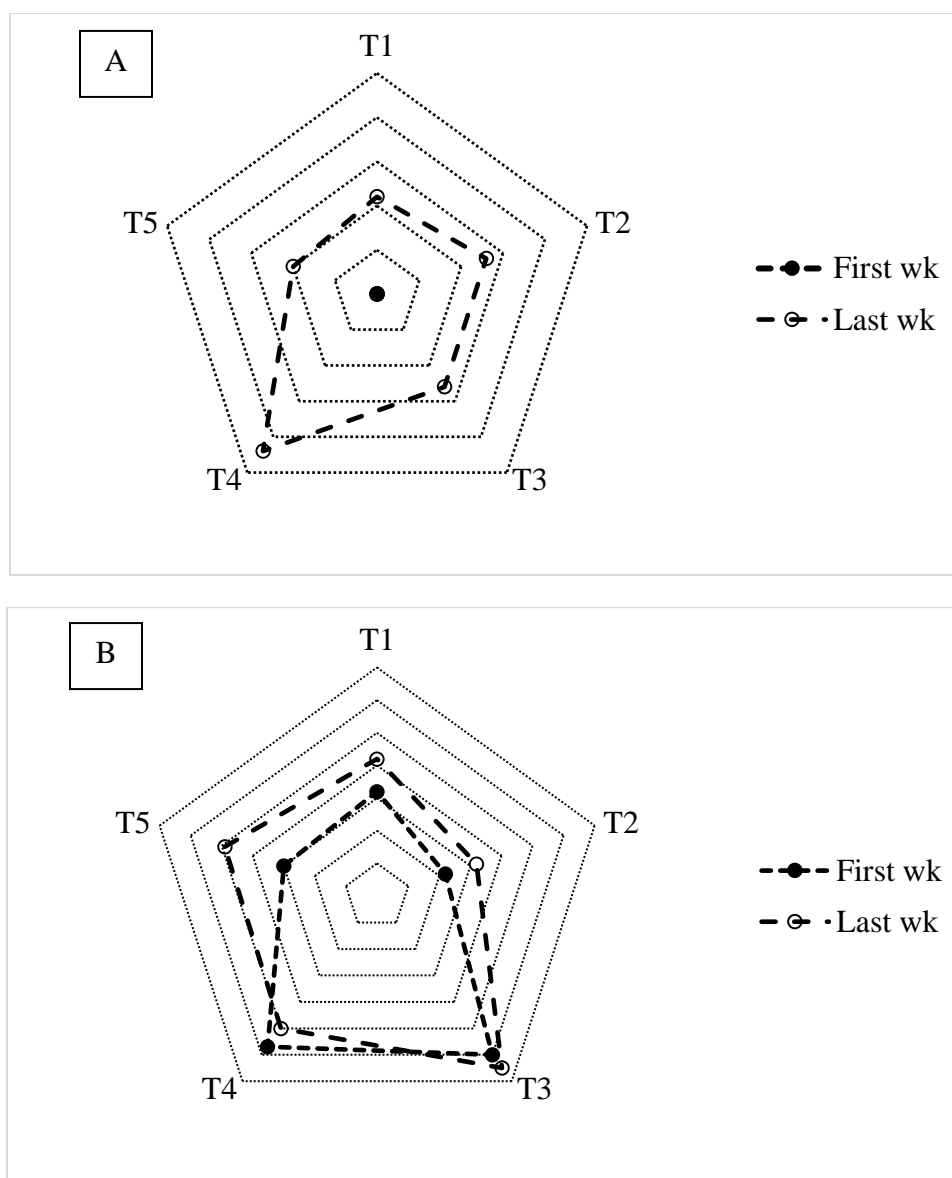


Figure 4. Descriptive sensory analysis of cottage cheese on a 0 to 15-point universal intensity scale indicating, A) the level of bitterness, and B) Firmness of cottage cheese at the first and last week of storage at 4°C. T1: Cottage cheese made without added any spore formers and rennet, T2: Cottage cheese mixed with the cream dressing added with proteolytic spore former, T3: Rennet added cottage cheese but without added any spore formers, T4: *B. subtilis* DBC added cottage cheese, T5: a cocktail of *B. subtilis* DBC and *P. polymyxa* DBC1 added cottage cheese, n=3.

OVERALL CONCLUSIONS

Many researchers have documented the prevalence of spore formers in dairy environments and their spoilage-causing activities in dairy products. Some of them had reported influenced texture and developed off-flavors in cultured dairy products such as psychrotrophic proteolytic spore formers, but none of them had studied how they affect the product quality. Contamination of spore formers after fermentation is most likely does not influence the final product quality because of the low pH, but their contamination just prior to fermentation may bring potential changes in final product quality. Therefore, we hypothesize that although the number of spore formers or spores in the final fermented product may be low, and their growth during refrigerated storage may be limited, the spoilage-causing activity of spore formers during first few hours of fermentation until the pH is low enough to inhibit their growth would cause the sufficient changes during fermentation which may influence the quality and functionality of the final product.

In our first study, we have evaluated various spore-forming strains isolated from dairy environments such as raw milk, biofilms formed on heat exchangers, processing lines and equipment, and finished product such as milk powder for their ability to degrade milk protein, milk fat, phospholipid, common stabilizers (gelatin, starch, xanthan gum, and pectin) utilize in the manufacture of yogurt and sour cream, and exopolysaccharides at typical sour cream and yogurt fermentation temperatures. The order of susceptibility to the degradation of tested components to isolates was starch > xanthan gum > protein = gelatin > phospholipid > pectin > fat at sour cream fermentation temperature (24°C), and starch > protein = gelatin > xanthan gum > phospholipids >

pectin > fat at yogurt fermentation temperature (42°C). Moreover, proteolytic strains with a higher level of proteolysis had degraded casein, primarily β - and κ - CNs which can cause texture or flavor defects in sour cream and yogurt. With the current demand for clean label dairy products, some of the cultured dairy products are fermented with exopolysaccharides (EPS) producing starter cultures. EPS produced during fermentation acts as a natural stabilizer which can eliminate the need for added stabilizers. We have tested spore formers to degrade EPS produced from commercial and sour cream starter cultures. Selected spore formers were also capable of degrading exopolysaccharides produced from the common commercial yogurt and sour cream starter cultures. The findings of this study should be of interest to the dairy industry because of the prevalence of stabilizers and EPS-producing cultures used in sour cream and yogurt. Our observations indicate that the presence of spore-forming bacteria found in dairy environments may negatively impact the texture or flavor of fermented dairy products.

In our second study, we have inoculated the common dairy spore-forming spp. such as *B. subtilis* DBC as the proteolytic strain, and *B. licheniformis* DBC1 as the proteolytic, lipolytic, and phospholipids degrading strain separately and their cocktail at 10^3 cfu/mL in sour cream mix just prior to fermentation and studied their influence on rheology, texture, physicochemical, sensory, and microstructure of sour cream. This study indicated that contamination of proteolytic spore-forming *Bacillus* spp. during fermentation induced the limited level of proteolysis resulting in inferior texture which can cause the batch to batch variations during manufacturing of sour cream. There wasn't any influence was observed by lipolytic spore-forming *Bacillus* strain used in this study. To our knowledge, this is the first study to show the impact of spore-forming *Bacillus* on

sour cream texture. Strategies to control spore-forming *Bacillus* in sour cream need to be developed.

In our third study, we have inoculated proteolytic spore formers separately and their cocktail at 10^5 cfu/mL in milk just prior to fermentation and in cream dressing and studied their influence on cottage cheese properties. Results demonstrated that proteolytic spore formers could influence the cottage cheese texture, yield and produced more grit. Their proteolytic activity can develop bitterness in cottage cheese. This study indicates that cottage cheese made from milk contaminated with proteolytic spore-forming strains can negatively impact the yield, curd size distribution, physicochemical, textural, sensory properties, and the shelf life. Strategies to control spore formers in cottage cheese need to be developed.

Curricular Practical Training (CPT) Report

I am pursuing Ph.D. in Biological Sciences with specialization in Dairy Science. While conducting research projects during my doctorate program, I realized that being an international student, it is very important to gain the advanced knowledge from dairy industry in the USA which could potentially give me the insight while conducting my research projects. I discussed my research projects need to my academic advisor and requested for curricular practical training (CPT) to Office of International Affairs at South Dakota State University. The knowledge and the industrial approaches that I have learned during my CPT are described in a report.

Introduction

On my CPT, I privileged to gain the practical knowledge from one of the top dairy leaders in the USA in the area of ice cream and frozen desserts. I have gained knowledge and experience in many aspects during my CPT such as the impact of milk proteins on the texture of high protein ice cream, importance of ice cream rheology, microbial quality of ingredients and food safety, etc., but I am restricted to provide the information on the sensitive projects because of the company's confidential policy. In this report, I would specifically like to discuss one of the projects in which I was involved during my training period, which was on the application of rheology in the process development of strawberry and chocolate fudge revels at their plant facility. In general, revel is defined as the chocolates, fudges, caramels, peanut butter, marshmallows, fruits, etc. with a flowable consistency which is usually mixed with ice cream to give typical attributes. For example, strawberry revel in strawberry flavored ice cream.

Opportunities and challenges

Company was outsourcing strawberry and chocolate fudge revels from the suppliers which were high in cost. These revels utilize in many different ice cream varieties and company consumes these revels in a high volume. Company's management had decided to produce these revels at their plant facility as the cost reduction opportunity. The biggest challenge for in-sourcing these revels is to optimize their consistency for further application, therefore the understanding of their rheological properties is essential.

Methodology

Manufactured in-source strawberry and chocolate fudge revels were continuously analyzed for their rheological attributes to optimize their consistency. The data set of 30 of rheological analysis was collected for each revel.

Rheological programs for revels:

1) Strawberry revel:

Instrument: Black Pearl Rheometer, ATS Rheosystem

Program: Shear ramp from 1-300 s^{-1} delay for 2 s with 5 s integration time at 7°C,

Concentric cylinder-25 mm.

2) Chocolate fudge revel:

Instrument: Black Pearl Rheometer, ATS Rheosystem

Program: Shear ramp from 1-300 s^{-1} delay for 2 s with 5 s integration time at 7°C, a parallel plate (30 mm) with 1 mm gap.

Results

Strawberry and chocolate fudge revels were observed with different rheological properties. Strawberry revel was observed with the highest viscosity of 75.9 cP whereas the lowest viscosity of 1.10 cP was observed from 1 to 300 s^{-1} within the collected data set (Fig. 1). Chocolate fudge revel was observed with the highest viscosity of 114.8 cP whereas the lowest viscosity of 1.9 cP was observed from 1 to 300 s^{-1} within the collected data set (Fig. 2). Chocolate fudge revel had a thicker consistency as compared to strawberry revel. Both revels had shown shear thinning behavior (reduced in viscosity with increased shear rate). No change in viscosity was observed after certain shear rate values in both revels.

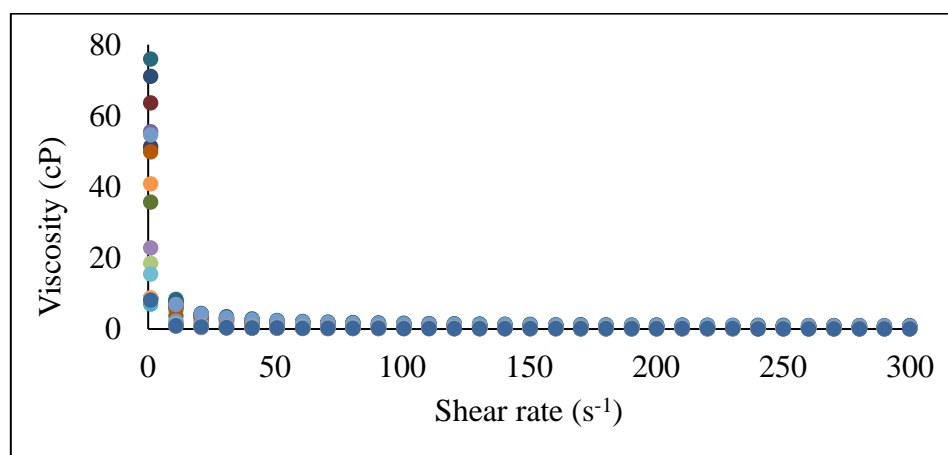


Figure 1. Rheological analysis of strawberry revel

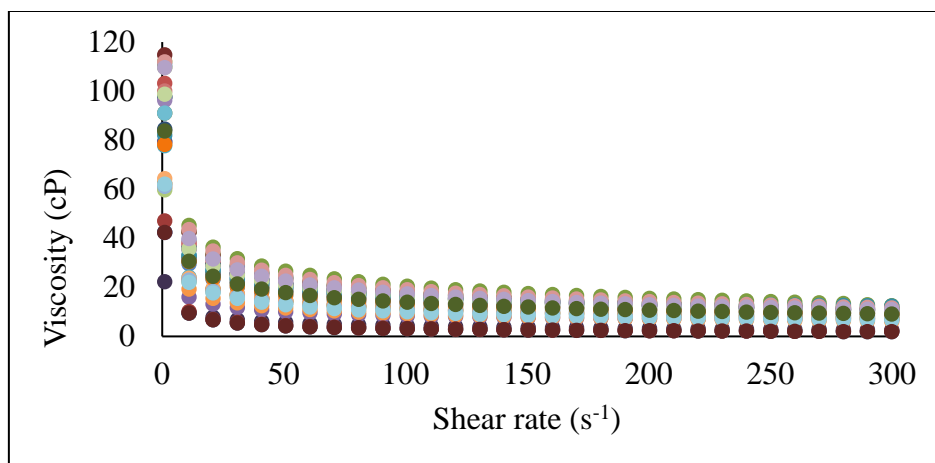


Figure 2. Rheological analysis of chocolate fudge revel

Conclusion

Strawberry and chocolate fudge revels were observed with a shear thinning behavior with a constant viscosity after some shear rate values.

Rheological analysis helped to define the specifications for the acceptance of these revels to get their optimized consistency. Manufactured revels must need to pass these specifications before their further application in ice cream and frozen desserts.

Specifications:

1) Strawberry revel:

Instrument: Black Pearl Rheometer, ATS Rheosystem

Program: Shear rate: 100 s^{-1} delay for 30 s at 7°C , Concentric cylinder-25 mm.

Viscosity: 1.2 to 2.0 cP

2) Chocolate fudge revel:

Instrument: Black Pearl Rheometer, ATS Rheosystem

Program: Shear rate: 100 s^{-1} delay for 30 s at 7°C , a parallel plate (30 mm) with 1 mm gap.

Viscosity: 8.0 to 22 cP